

Where next with the greenhouse?

The US Congress seems to have taken the threat of catastrophic greenhouse warming under its wing politically. But there is a long way to go before it can decide what to do about it.

Now that the seasonal summer has at last arrived in the Northern Hemisphere, it is natural enough that the US Congress should be in turmoil (see p.714) about the greenhouse effect, a phenomenon that comes to the surface every few years. But it is hard to believe that either of the two representative houses that sit on Capitol Hill in Washington can seriously think they have the means to legislate about this complicated matter when, to tell by recent experience, they cannot bring themselves to do the decent thing by the government of which they are a part, and avoid spoiling the administration's posture on free trade if the result is that they must abandon protection for US printing.

Here are a few homilies on the greenhouse effect that may (but that is unlikely) give Congress pause. They are offered, so to speak, in a spirit of moderation: Congress is indeed worrying about an issue on which the expenditure of anxiety is appropriate and even potentially constructive, but there is a danger that it may pace its anxiety by the timetable laid down for general elections, every two years for mere representatives and six years for grander senators. The time-span of the greenhouse effect is longer than either. And it is incalculable.

Recent past experience shows that well enough. Carbon dioxide absorbs radiation more efficiently in the infrared than in the visible part of the spectrum, so that if it should accumulate in the atmosphere, the result should be that solar energy will the relatively more easily reach the surface of the Earth than escape from it, increasing the surface temperature. This primitive scenario has been current for the best part of half a century. Moreover, it is now plain that carbon dioxide has indeed accumulated in the Earth's atmosphere, by roughly 30 per cent in the past century. So the temperature at the surface of the Earth should be steadily increasing, should it not?

Events have unfortunately taken a more tortuous course. The increase in the concentration of atmospheric carbon dioxide has been substantial and dramatic, but is roughly only a half of what it would have been if all the fossil fuel burned in the past century had produced combustion gases that had stayed in the atmosphere. Where has the rest gone? A general flourishing of vegetation is one possibility, solution in the oceans is another. There is also the curious but surprising conundrum that, the concentration of carbon dioxide having increased, the temperature has not followed by the amount predicted. The difficulty here is that the soothsayers (many of them professional scientists) have made more of their first-approximation models than is proper; they may not, for example, have disclosed that they cannot cope with real clouds in their climatic models or that ocean-atmosphere interactions are accounted for only in the most rudimentary way, but are likely when properly counted to be stabilizing. Even so, the long-term outlook cannot but be gloomy. One day, in the next few decades or so, the greenhouse effect will become reality. That, with its occasional introspections on the subject, is the issue for which Congress is preparing itself.

It might do worse than read the two articles on the remarkable disappearance of ozone from the Antarctic which appear on pp.755 and 759 of this issue. Ozone is an important component of the lower stratosphere because it absorbs solar ultraviolet,

heating that layer and protecting the people who live beneath from ultraviolet radiation that might cause skin cancer. What the data show is a quick destruction of atmospheric ozone when the Sun appears above the horizon at the end of the Antarctic night; the assumption that most damage to the stratospheric ozone layer is done by chlorine is on this occasion complemented by the damage done by bromine, another halogen used for the general good in the putting out of fires, whose use is generally applauded by Congress. These phenomena also have an influence on what the greenhouse will do, but in ways that cannot yet be determined. The importance of these components of the upper atmosphere is that their presence has been recognized only recently, that they may in various chemical guises be greenhouse gases like carbon dioxide, but that their influence may also tend in the other direction.

What should Congress do? First it must recognize that the problem is essentially international. Everybody puts carbon dioxide into the air, for reasons that are usually good — keeping warm, cooking food, getting from one place to another. So when the temperature of the Earth's surface begins significantly to increase, some mechanism will have to be invented whereby something that might provisionally be called the World, or the World Community, is empowered to tell smaller parts of itself that they cannot continue doing what they have always done.

While the general principle is generally acceptable, its implementation is beyond the present ken of those who generate carbon dioxide. A host of academic conferences in the past decade has amply illustrated what the difficulties would be. Should carbon dioxide (or other greenhouse gas) quotas be proportional to present GNP? Or to potential GNP? Or to some other index of a country's ambition, such as the size of its population? By what means could such an agreement be enforced? By fines proportional to the disadvantage done to the peoples of countries other than the transgressor's? And should the disadvantaging multiplier be so great that people would find it more profitable to turn to nuclear power than to pay the fines? The enthusiasts for the greenhouse problem now raising the roof in Congress had better take a close look at what lies down the pike before they pin their electoral futures on this scary prospect.

What is to happen to the rest of us? In a world in which legislative bodies, including Congress, are notoriously unwilling to sign binding international agreements, there are two rational attitudes to strike. One is to concentrate on the uncertainties in present calculations, and on the features of most respectable calculations which show that there will be winners as well as losers in the global greenhouse, and to say that the problem is of no significance. The other is to take the line that there will be a greenhouse problem one day, perhaps next decade, perhaps a century from now; in that case, the rational course is now to seek to strengthen international institutions, and the trust that ordinary people put in them, against the time that a durable agreement is needed. Sadly, there is no obvious correlation between the opinions on contemporary international issues among those who have lately rediscovered the greenhouse effect and the small membership that would, for example, do something about arms control. □



Atmospheric greenhouse

US Congress says climate is part of politics

Washington

POLITICAL efforts are under way in Washington to bring into the policy arena the depletion of the atmospheric ozone layer warming due to the greenhouse effect. Declaring that the topics "can no longer be treated solely as important scientific questions", Senator John Chafee (Republican, Rhode Island), chairman of the Senate subcommittee on environmental pollution, last week announced that he would ask the Environmental Protection Agency (EPA) and Congress's Office of Technology Assessment to conduct studies of policy options to stabilize world levels of atmospheric gases.

Chafee's statement was made at the start of two days of hearings at which administration officials and prominent scientists were called to give evidence on the problem and their views of appropriate responses. Chafee also said he would ask the National Academy of Sciences to review gaps in scientific knowledge on the topic, and that he would ask the State Department to bring the issue to international attention.

Although administration officials at the hearings generally stuck to the line that more research is needed before policy

options can be considered, EPA administrator Lee Thomas said a working group has been established within the agency to estimate trends in greenhouse gas emissions and to determine possible control options. The working group will also conduct long-term research.

EPA is already considering further controls on the use of chlorofluorocarbons (CFCs), which catalyse the breakdown of the ozone layer; a decision will be made by November 1987. Several witnesses referred to recent measurements showing a 40 per cent reduction in the ozone layer over Antarctica, the causes of which remain unknown but which may be related to human activities.

Concern over the effects of CFCs on the ozone layer led the United States in 1978 to ban their use in non-essential aerosol sprays, but EPA has failed to persuade other nations to follow suit. Thomas said EPA had "stepped back" from those efforts, but it is considering regulations further to limit domestic uses of CFCs, used mainly as refrigerants.

Several witnesses at last week's hearings referred to the need for international co-operation on research, including Thomas and Alvin Trivelpiece, director of the

office of energy research at the Department of Energy (DoE). DoE is concluding an agreement on joint climate research with China, to be conducted through the Chinese Academy of Sciences. Other countries are participating in DoE's carbon dioxide research programme.

A scientific view of the problems was provided by James Hansen of the National Aeronautics and Space Administration (NASA)'s Goddard Space Flight Center. Hansen has used a global climatic model to simulate the climatic effect of the transient growth of atmospheric carbon dioxide and other trace gases now thought collectively to be as important.

Among Hansen's results is the conclusion that a large part of the equilibrium warming due to the well-documented carbon dioxide increase since 1850 has probably not yet occurred; the same is true for the other gases. The effect of warming will start to emerge from statistical background noise in the 1990s.

In response to submitted questions, Hansen also prepared estimates of the increased number of days per year over 90 degrees Fahrenheit in Washington DC that would result from a doubling of carbon dioxide. Conclusion: from 35 days (at present) to 85, with 12 of them over 100 degrees. Temperature across much of the United States might rise by an average of 3 degrees Fahrenheit or more by 2020. Local press, sweltering in 90-degree-plus weather, were duly impressed.

Hansen said later in an interview that he was surprised at Chafee's call for immediate studies of policy options; he would prefer to emphasize the need for integrated and long-term studies of climate systems as he thought policy options would not meet political approval in the absence of clearer evidence. Recommendations along these lines have been made in a report to be published by the NASA Earth System Sciences Committee chaired by Francis Bretherton of the National Center for Atmospheric Research. But, while also stressing the need for more research, Bretherton said he approved of Chafee's call for an assessment of policy options.

Although the greenhouse effect is not at present under specific consideration in the White House, a consensus is emerging in the scientific community on the need for coordinated and long-term measurements of climate. An international initiative has been proposed by the International Council of Scientific Unions, and the roles of US federal agencies in a long-term climate study are being considered in the administration by the Senior Inter-agency Group on Space, called SIG-Space. A major international conference on the subject sponsored by the United Nations Environment Programme and EPA takes place this week in Arlington, Virginia.

Tim Beardsley

French universities

One reform too many?

FRENCH university presidents walked out of a meeting with the new research and higher education minister, Alain Devaquet, last week, after hearing that France is to abandon its newly-created PhD degree, much welcomed by natural scientists for its similarities to the research degrees of Britain and the United States. The plan is to return to the old idiosyncratic system of "troisième cycle" and "thèse d'Etat" degrees.

This is just one of the reforms promised last week by Devaquet, a physicist, in a new law for the universities to be put before the National Assembly before the summer. Another reform will allow universities to select their own students, the first such selection system allowed in France since the European university revolution of 1968. But the selection will be only nominal: Devaquet, no doubt fearful of the political consequences of avowing selection, has also reaffirmed the right of every school-leaver who passes the coveted "baccalaureate" to gain a university place, just as each of them can do at present. It will be up to local education

authorities to square this selective circle.

Other promised reforms include a lightening of the system of the three elective councils by which universities and their departments are managed, in order to give more power to professors ("and less to cleaning staff", according to one supporter), but the present national system for awarding degrees will be retained.

The law thus does not go so far as the promise of Prime Minister Jacques Chirac to return autonomy to the universities both in the selection of students and the awarding of degrees. But it went far enough for the university presidents to walk out of their meeting with Devaquet, which was held just a couple of hours before the minister's previously arranged press conference on the new law. The presidents were protesting at an almost total lack of consultation in the brief preparation of the law since the general elections in March, and at the prospect of yet another long hot summer of discontent as the French university system is yet again lifted up by the roots to see how the fragile plant is growing.

Robert Walgate

US copyright

Will Congress blot US copybook?

Washington

WHILE the US administration bangs the drum of free trade, Congress is busily preparing to muffle its beat. Now, a coalition of 54 major corporations and association is lobbying hard to persuade Congress not to extend legislation requiring non-dramatic works by US authors to be printed in the United States.

The so-called "manufacturing clause" has been declared illegal by the General Agreement on Tariffs and Trade (GATT), and the European Economic Community (EEC) has threatened economic sanctions against the United States if the clause is not allowed to expire at the end of this month. But influential members of Congress want the clause made permanent, and the Senate Finance Committee has approved a bill to do so.

The current manufacturing clause prohibits importing into the United States copyrighted non-dramatic literary material in English unless it is printed and bound there. The effect is to ensure that US copyright material is printed in the United States. The price of books in the United States is consequently estimated to be 12 per cent higher than it would otherwise be. Newspapers are also affected.

Among those most affected by the bill are manufacturers of computers and business equipment; the legislation prevents them from printing instruction manuals and the like abroad, where many of their manufacturing plants are situated. Burroughs, IBM and Xerox are among the corporations that have joined the Coalition to Oppose the Manufacturing Clause. EEC estimates the clause costs it between \$300 and \$500 million per year and has threatened retaliatory sanctions.

The clause is also opposed by the US Department of Commerce: commerce secretary Malcolm Baldrige told the Senate Finance Committee last week that the clause is "an embarrassment" that undermines the United States' position as it tries to negotiate fair trade practices and strong protection for intellectual property rights overseas. Baldrige said he would advise President Reagan to veto any legislation Congress might pass to extend the clause.

The clause was formerly set to expire in 1982, but Congress voted then to extend it and overturned a presidential veto. It had not by then been declared illegal by GATT, however, and opponents are using the GATT ruling as a major plank for their case. The clause also makes it impossible for the United States to adhere to the Berne copyright convention, which provides international protection for intellectual property rights.

The controversial clause dates back to 1891, when it was enacted to protect the

US printing industry. Although it has since been progressively weakened, the printing industry would still like to see the clause extended, and some members of Congress are unwilling to be seen to be increasing foreign competition for the industry. Critics contend, however, that the number of US printing jobs that would be saved if the clause is extended is trivial compared with the number of jobs lost in other export industries. Singapore, thought to be one of several countries that turn a blind eye to piracy of US-copyright trade materials, has tied improvements to its copyright laws to an exemption from the US manufacturing clause, and other countries may follow suit.

Some computer manufacturers have already chosen to forgo US copyright protection on their manuals printed overseas rather than print them in the United States, according to the coalition. Vico Henriques, president of the Computer and Business Equipment Manufacturers' Association, said it was recognized good business practice to print documentation for a product at the point of manufacture so that local alterations and conditions could be included easily.

The legislation approved by the Senate finance committee to extend the clause (S. 1822) would remove the existing exemption for Canada. The exemption allows Canada to export to the United States US-copyright works not printed in the United States. Retaliatory action from Canada would therefore also be on the cards if the legislation were passed.

To placate the opposition, the Senate bill, introduced by Republican Senator Strom Thurmond, includes some compromise provisions. The chief one is that after two years, waivers could be issued allowing imports from countries which the Secretary of Commerce certifies as fair trading partners that respect US copyright laws (among other things). But the US Trade Representative, Clayton Yeutter, told the finance committee that he had "strong doubts that Canada, the European Economic Community or Japan could obtain a waiver" under the "cumbersome" waiver provisions. Yeutter said the ostensible purpose of the clause, protection of US printers' jobs, was unnecessary: total employment in the US printing and publishing sector is projected to grow by 2.2 per cent a year to 1995, creating more than 30,000 jobs each year.

The House of Representatives' Judiciary Committee is soon to consider a bill identical to Thurmond's. Senate floor action on Thurmond's bill is scheduled for next week; the coalition is only cautiously optimistic that it will have rallied sufficient votes to defeat it.

Tim Beardsley

British software

Looking over the shoulder

THE common British conceit that Britain may not be much good at hardware, but has the edge in software, takes a tumble this week with the publication of a report by a committee of the Advisory Council for Applied Research and Development (ACARD) that cries for urgent action from everybody in sight (*Software: A vital key to UK competitiveness*, HMSO, £6.00). It is not true, says ACARD, that British software houses run on an inside track; after the United States, France is next, while even in Europe, West Germany is number two.

Both perceptively and courageously, ACARD sees the British software problem as part of the general decline of British industrial competitiveness. While the "man in the street" may still expect that software houses would be among the most rapidly growing and most profitable parts of British industry, the real prospect is quite different.

The committee says that evidence from overseas confirms its own view that the rate of growth of the British software industry will lag behind that of the world leaders, that market shares will decline and that the British market for software will be dominated by overseas suppliers in the 1990s.

The ACARD committee gives several reasons for this gloomy outlook, including the chronic shortage of skilled people in information technology, the failure of companies in the field to spend enough on selling the products they have developed and, latterly, their unwillingness or incapacity to invest in research and development. According to the ACARD report, the chief objective among software houses now is to make software development less labour-intensive, which means investment in the techniques of automation.

The diagnosis is more persuasive than the remedy. Above all, ACARD wants Britain to know there is a problem. Beyond that it has a miscellany of remedies, such as the proposal that government departments should give thought to generic needs when commissioning new developments, that the Science and Engineering Research Council should elevate the information engineering committee of its engineering board to the status of a full board and that the programming language BASIC should be outlawed as soon as it can be replaced by something better.

The committee's theme is that designing a piece of software is like making anything else — a task in engineering in the widest sense. Many British software-users will say amen to that. □

Cancer research

Boom in new laboratories

FUNDAMENTAL cancer research in Europe is about to be strengthened by several new privately financed laboratories. One will be in Vienna and is jointly owned by Genentech, the Californian biotechnology company, and the West German chemical and pharmaceutical company Boehringer Ingelheim. The other four, and probably five, laboratories are new branches of the Ludwig Institute of Cancer Research, administered from Zurich.

Despite its commercial backing, the Vienna institute will be concerned only with basic research in the general area of molecular pathology, with a particular focus on oncogenes. First rights on any discovery of commercial interest stemming from the research are shared by Genentech and Boehringer Ingelheim. The German partner will foot the bill for the institute for its first five years, after which costs will be shared equally.

During the initial five-year period, Genentech's contribution is know-how, in terms of both the recruitment of suitable staff and the structure of the research programme. Just over half the cost of building the institute (in Vienna's third district) and 80 per cent of the running costs for the first five years will be met by Boehringer Ingelheim, with the balance coming from the city of Vienna and the Austrian government, eager to counter a Freudian past with a double-helical future. Various relevant university institutes, at present scattered around the city, are likely to be re-sited alongside the new institute.

Building work on what is likely to be called the Institute of Molecular Pathology will begin this summer. Shortly thereafter its director, Dr Max Birnstiel, will leave the University of Zurich for Vienna. Negotiations are already well advanced with some potential group leaders for the institute and enthusiasm for heading east is running high.

Those who prefer to head west or north may find positions in four new branches of the Ludwig Institute of Cancer Research. Two of these have begun operation in Sweden (in Stockholm, under Dr Ulf Pettersson and in Uppsala, under Dr Carl-Henrik Heldin) and two will start soon in London (at Middlesex Hospital Medical School, under Dr Michael Waterfield, and at St Mary's Hospital Medical School, under Dr Paul Farrell). Negotiations are under way for another London branch and Dr Webster Cavenue has just begun to direct a new Ludwig in Montreal's McGill University.

As a result, there are now 14 Ludwig branches consuming US\$33 million last year. There will now be a period of consolidation with no new branches, according to Mr Hugo Frei, chairman of the

board of directors, which is responsible for managing the assets provided by Mr Daniel K. Ludwig in 1974 in such a way as to produce sufficient income to finance the research programme. Each branch, of 20–80 people, is set up in cooperation with a hospital for an initial six-year period and is subject to review every three years. So far, the only branch to be closed was the previous London laboratory at Sutton (see *Nature* 318, 400; 1985) for

reasons that included its location, lack of patients and scientific shortcomings, according to Frei.

To judge by the new branches, Ludwig has little difficulty in attracting good directors despite the lack of guaranteed long-term support and a policy that sets salaries at local levels.

There is, however, some generosity in terms of capital expenditure. A recent board decision means that the US Research Corporation will handle the transfer to commerce of any potentially profitable discovery in a Ludwig laboratory.

Peter Newmark

Soviet education

University shake-up canvassed

A RADICAL reorganization of Soviet higher education is on the cards. In what is called a draft proposal, published last week, the Central Committee of the Soviet Communist Party says the time has come for curricula to be "retuned" and for the administration of higher education to be simplified. There should be more money for equipment and better salaries for professors. If implemented, the proposals will give universities a more prominent place in the pattern of Soviet research, at present dominated by academy and ministry institutes.

The theme of the proposals is familiar outside the Soviet Union, to arrange matters so that higher education contributes more fully to the national economy. But the breadth of what is proposed represents radical reform of many established practices and institutions.

The scale of Soviet higher education, with a total of 894 universities and other institutions of higher education, is huge. But, according to the Central Committee, the system is bedevilled by bureaucracy. Part of the trouble is the division of responsibility between the All-Union and republic governments, but ministries and departments also run their own institutions for training people with special skills, as in agriculture and medicine. Altogether, 74 ministries and departments are in some way involved, but 30 of these run only one or two institutions (called VUZy when they are not universities).

To meet the complaint that many production ministries and departments fail to provide suitable teaching staff and facilities for their institutions, the Central Committee proposes that the Ministry of Higher Education should have more control of VUZy run by other ministries. There would be regular checks of teaching standards and, because sectional interests have often led to the duplication of specialist courses and, sometimes, whole new VUZy, the system would be rationalized so that redundant courses and establishments would be gradually phased out.

Teaching is also to be overhauled. The Central Committee says that the present pay structure discourages the best scholars and scientists from making an academic career. It says that too much emphasis is placed on formal lectures and textbook knowledge, with the result that students are overworked, while the ratio of students to staff is far too high.

What the Central Committee wants to see is more teaching in small seminar groups (not more than 15 people), with more practical and laboratory work. The notion that each student should have an individual timetable and more choice of lecture courses is encouraged, and there is even a tentative suggestion that intending university teachers should spend a probationary period of two years in some part of the national economy in which their students may later work.

Recruitment into graduate programmes will centre on people seconded from industry or with work experience in their chosen field. Research for the degree of Candidate (PhD) will concentrate on "priority fields of science and technology" and efforts will be made to raise the ideological as well as the scientific standard of research students. The possibility is canvassed that some universities and VUZy might offer courses leading to the degree of Doctor of Science, at present awarded on the basis of a second dissertation in the course of a professional career.

Apart from the threat of more direct control, the Central Committee's prospectus should be welcomed by Soviet universities and VUZy. More is to be spent on laboratories, libraries and computers. Students are to have better living conditions, and academics better pay. There is even talk of higher aesthetic standards for university buildings. In return, the universities and VUZy have to produce graduates of the type required by the "scientific and technical revolution" and also play a part in the intended "unified state re-training scheme for older generations of scientists".

Vera Rich

Chemical weapons

Binary weapons in trouble

Washington

A GENERAL Accounting Office (GAO) report has given a black eye to Bigeye, the binary chemical weapon the US Department of Defense (DoD) says will give the chemical weapons arsenal a deep-strike capability now said to be lacking. But in spite of the doubts raised by the GAO report, DoD is proceeding with operational tests of Bigeye, and hopes soon to begin initial production at a low rate.

The GAO report focuses on DoD's tests of Bigeye performed since 1982. The Bigeye bomb consists of two non-lethal chemicals carried separately. When mixed, these agents form the persistent lethal nerve agent VX. Originally, DoD planned that high-flying aircraft would drop the bomb after the two chemicals had combined. But lessons learned during the 1976 Yom Kippur War in the Middle East convinced DoD that this approach made planes too vulnerable to anti-aircraft missiles, so that DoD decided that the bomb would have to be carried by aircraft flying at high speed and low altitude.

This plan also has problems. The extra temperature caused by increased air friction at low altitudes raised pressure inside the bomb, causing one to explode during testing. This is why, since 1982, DoD's plans for delivering Bigeye have required mixing the two chemicals only after the bomb has left the plane, and "lofting" the bomb — tossing it upwards from the aircraft — so that the chemicals have time to mix before the bomb's contents are released. The GAO report concludes that this delivery scheme places a heavy burden on pilots and systems software.

Eleanor Chelimsky, one of the report's principal authors, said at a press conference last week that the Bigeye bomb has satisfied neither chemical purity requirements nor system reliability, and is "therefore not ready for production". Pressure build-up inside the bomb after mixing could still result in a premature release of its contents. DoD solved the pressure problem in laboratory tests with a pressure relief valve, but the valve will not be included in working models of the bomb.

Another concern is the potential of the bomb's chemical components to catch fire or "flash", destroying the nerve agent's effectiveness. Also troubling Chelimsky is the way DoD performed tests on the bomb. "DoD seems to be confused about what in fact has been tested and what the results have been", she says. According to the GAO report, DoD changed performance criteria during testing to make Bigeye look better.

DoD is quick to defend Bigeye. John E. Krings, director of DoD's office of Operational Test and Evaluation, agreed that

GAO had identified some of Bigeye's initial problems, but said the report was "not totally accurate". Krings denied that the criteria that the weapon had to meet had in any sense been eased, pointing out that all new weapons systems experience modifications in design criteria during testing. The bottom line, says Deputy Assistant to the Secretary of Defense for Chemical Matters Thomas J. Welch, is whether the bomb represents an improvement in combat effectiveness. Welch believes it does. "We understand the shortcomings of Bigeye", says Welch. "We have a handle on fixing them, and the fixes are under way." The logic of binary weapons is that they are safer to store and transport than unitary weapons. But even here Bigeye has problems. VX is formed by mixing QL (ethyl 2-(diisopropylamino) ethyl methyl-phosphonite) with rhombic sulphur. But according to sources at GAO, DoD is aware that if QL were accidentally released near a power plant burning high-sulphur coal, VX could form spontaneously.

Bigeye is just one part of a planned improvement in chemical weapons. By 1994, DoD plans to destroy all existing stocks of unitary weapons, switching over to binary weapons. Besides Bigeye, DoD plans to deploy 155-mm artillery shells carrying the non-persistent nerve agent GB, and a multiple-launch rocket system also carrying GB. But Congress has required that the North Atlantic Council, the political arm of the North Atlantic Treaty Organization (NATO), should approve the United States' "force goals" before production can proceed. Congress has also insisted that NATO's Supreme Allied Commander in Europe must establish plans for how chemical weapons will be used before production funds are released.

To gain European support, President Reagan is said to have struck a deal with West German Chancellor Helmut Kohl to remove existing stockpiles from West Germany in exchange for Kohl's support for binary weapons production. Last month, the NATO Defense Planning Committee endorsed the US plans for new chemical weapons production, a move the administration hopes will allow production to proceed. But congressional opponents argue that North Atlantic Council approval is still needed.

Even if the binary weapons plan is approved, critics worry that not having the weapons actually based in Europe will make them ineffective as a deterrent. DoD plans to airlift the bomb components separately to Europe in the event of an attack. The United States holds to the position that it will never be the first to use

chemical weapons. But critics worry that a massive influx of such weapons to Europe at a time of crisis may make it appear that the United States is abandoning that policy, prompting a Soviet first strike.

A report by the specially empanelled Chemical Warfare Review Commission to President Reagan last year concluded that the long-range capability offered by the Bigeye bomb is urgently needed if the United States is to have a credible chemical weapons deterrent. But many in Congress believe the binary bomb is just an expensive mistake. The GAO reports should provide congressional opponents with ammunition when they attempt to shoot down Bigeye.

Joseph Palca

Estonian protest

A GROUP of Estonian scientists, including members of the Estonian Academy of Sciences, has written an open letter to the West, calling for protests against Soviet plans to build a large oil terminal at Muuga near Tallinn. The terminal, which will be adjacent to the controversial grain harbour, will, the scientists say, be a major source of pollution to north Estonia and to the whole Baltic area. The oil terminal, although necessary to the All-Union plans, is not necessary to the economy of the Estonian republic. Since the plans for the terminal have not been officially announced (the scientists describe their source as a reliable leak from civil servants), they clearly hope that international criticism might induce the central planners to scrap the project quietly, and to deny the report as unfounded.

The area under threat by the proposed terminal includes lake Peipsi, the main water source for Tallinn. It is the only area of the country not already a victim of major pollution from oil-shale and phosphorite mining. (A recent study by the Estonian Academy's Institute of Economics found that the large phosphorite mine now being planned at Toolse will cause extensive seepage of pollution through the ground-water.) A particularly worrying aspect of the problem is that, since January 1986, censorship control has become so strict that it is now virtually impossible to inform the general public about environmental hazards.

The scientists, however, are not concerned only with the pollution threat posed by the oil-terminal, grave though that is. All major construction and engineering projects in Estonia are accompanied by a major influx of Russian labour, which, in its turn, means the gradual linguistic and cultural Russification of the area. If this policy continues, the scientists say, by the end of the century, a broad zone of north-eastern Estonia, comprising about 60 per cent of the population of the republic, will be effectively Russified. Vera Rich

Biotechnology

Insect virus as super-vector?

Washington

AN insect virus that could become a second-generation recombinant vector is gaining a quiet following in biotechnology. The baculovirus is easily engineered and achieves both high synthesis rates and complex processing of recombinant products. Cetus, Eli Lilly, Genentech and 15 other companies are investigating the system, but a three-year-old Connecticut company called MicroGeneSys claims it has already foiled the competition.

Although he heads only 25 employees, founder and president Frank Volvovitz believes MicroGeneSys has invested more time, money and expertise in the use of baculovirus than any other company. Volvovitz culled six virologists from laboratories at the National Institutes of Health, Baylor College of Medicine and Texas A&M for his research team. The company will not disclose the yields it has achieved, but published reports contain examples of recombinant protein production nearing 700 mg per litre of culture within a few days of infection. This rivals the rates of monoclonal antibody production by hybridomas, the most efficient recombinant machinery known.

The system is based on a cell line established in the late 1970s from the pupal ovarian cells of the moth *Spodoptera frugiperda*. When infected with baculovirus carrying a foreign gene, these cells can secrete recombinant products complete with post-translational modifications such as phosphorylation and glycosylation. The system may therefore dispense with costly downstream processing. Mammalian cell cultures offer the same processing advantages, but are not as prolific.

The productivity of the baculovirus derives from the efficiency of the viral promoter of the gene encoding the protein called polyhedron, the sole component of a crystalline matrix that acts as a protective shield for viral particles existing outside their insect host. Caterpillars eat the matrix and release the particles, which then temporarily suspend polyhedron synthesis. When the caterpillar is near death, the virus resumes matrix protein production until roughly 20 per cent of the larval host consists of polyhedron.

MicroGeneSys has already harnessed this productivity for a hepatitis-B vaccine which will enter clinical trials this fall. The company is also developing vaccines against acquired immune deficiency syndrome and malaria infections. Meanwhile, research in Texas A&M and the University of Idaho is generating a network of academic and corporate collaboration on baculovirus technology.

Eli Lilly has been working closely with Max Summers in Texas, who is generally

recognized as the technology's pioneer. Summer's laboratory is also working on interleukin-2 with Hoffman-LaRoche. Cetus is building on its preliminary results with colony-stimulating factor, while Genentech assigned a handful of researchers to investigate the system and says merely that it is "very interesting". Genetics Institute (which has just raised \$75 million in its first public offering of shares), had originally planned to use

baculovirus for making pesticides, but changed the emphasis of its programme on learning more about the system.

There are some sceptics in the field as well. Chiron considers that the baculovirus has only "marginal utility" compared with its own yeast systems. Even the staunchest supporters of baculovirology admit that the analysis of the gene products so far has not been thorough enough to ensure smooth passage through the regulatory process. Little is known, for example, about the characteristics of the insect cell glycosylation, which could affect biological activity. **Karen Wright**

European research

Still hope for budget increase

Luxembourg

RESEARCH ministers of the twelve member states of the European Economic Community (EEC) meeting here last week refused to triple the research budget of EEC, demanding that the European Commission, which administers EEC affairs, be more "realistic" in its requests. The Commission, however, regards its proposal for an ECU (European Currency Unit) 10,350 million (£6,600 million) "framework programme" of international research for 1987-91 as eminently realistic, and indeed vital for the economic future of Europe — and has retired to prepare a new proposal to put before the ministers in October.

But this apparently eyeball-to-eyeball confrontation is not official. It occurred over lunch — when no official minutes are taken. But the British, French and German delegations did not aid Commission digressions with their insistence on only small increases in the ECU 3,750 million five-year research budget of the EEC.

The EEC spend is dwarfed by the ECU 150,000 million or so that the individual nation members of EEC spend on research each five years, and the Commission is arguing, in effect, that a great part of this national spending is wasted, either by duplication of effort or by a failure of research or technological enterprises to reach a critical mass of talent or investment. In its strategy to 1991, the Commission aims to expand existing successful coordination programmes in areas such as information technology and biotechnology, and to turn pilot programmes into fully-fledged actions.

EEC research ministers, particularly in the larger European nations, are apparently holding back this expansion, or — even if the research ministers are more willing to support the Commission — over-zealous finance ministers in the newly monetarist France, in West Germany and the United Kingdom are putting the brakes on. Research ministers at a political level now strongly back the object-

ives of "improving Europe's technological competitiveness" and of increasing the "cohesion" among individual national research and technology policies, two of the current "buzz phrases" in European science policy, but at an economic level the ministers seem unable or unwilling to back the principles with cash.

Meanwhile, Geoffrey Pattie, the British minister of technology, recommended that the Commission could save money on science, by cutting its energy research by 20 per cent (it is set to remain roughly constant in the new framework programme) or by offering only fractional subsidies (down to 25 per cent on a sliding scale from the current 50 per cent) to university, institutional and industrial projects granted EEC support, thus making a small sum go further. The Chernobyl reactor disaster may make the first unlikely, as projected decreases in nuclear fission safety research may have to be reversed; and according to Commission officials the second is already practised.

Pattie and other "purse-strings" ministers present, including Alain Devaquet from France and Heinz Reisenhuber from West Germany, made it plain that when the Commission returns with new proposals they must be realistic or further delays in agreeing the programme are inevitable. Everything rests on what "realistic" means, but estimates were ranging here from around ECU 4,300 million — the sum estimated to be necessary to keep going with the present programme, to "around half" the proposed programme (some ECU 5,500 million). With these sums it would be possible to go ahead with the planned programme, according to Commission officials, but the number of grants awarded would be nowhere near able to satisfy the demand, the disappointment of good applicants would cause disillusion, and the function of the programme as a real catalyst of European economic and technological transformation would prove more difficult to achieve.

Robert Walgate

Materials research

Europeans wake up

Luxembourg

EUROPE is to launch an ECU (European Currency Unit) 30 million (£20 million) three-year programme of research on new materials, it was agreed here last week, a year after the proposal was first tabled. The programme covers ceramics to the new neodymium-iron-boron high-field permanent magnets which are posted to replace electromagnets and reduce the volume of electric motors by half. The plan has been held up only because the European Parliament demanded more than either the European Commission had proposed or the Council of Ministers, which takes decisions, could accept.

EURAM, (European Research on Advanced Materials), is a belated response to the spending of some \$1,000 million (£666 million) by the US Federal Government each year on materials science, to similar spending in Japan and to the relative weakness of European research and industry in new materials. In the new magnetic technologies alone, which were Japanese and American research discoveries just four years ago, General Motors has already begun pilot production of starter motors.

According to the Commission, "there are still excellent materials laboratories in Europe, but their innovative capacity is one the decline". EURAM aims to help reverse the decline, and also to strengthen perennially weak links between universities and industry. Its budget was less than the ECU 38 million the Commission had requested, but much more than what it had been spending in pilot programme.

In the ambitious "framework programme" also debated by ministers here (see p. 718), support for materials research would leap to some ECU 200–300 million over five years. Although the ministers declined to back the whole budget, a further expansion of the new materials programme won political support.

With the framework programme to be debated again in October, the Commission can meanwhile get on with implementing EURAM, which already has a detailed list of projects to support in metallic materials, engineering ceramics and composites. The object will be to link existing laboratories in joint programmes of research, with, preferably, an industrial partner. The programme will not be exclusive to the European Economic Community; the Swedish car-makers Volvo and Saab have already shown interest. Officials claim that 90 per cent of the leading European laboratories in the field of magnetic materials are involved in a pilot programme.

Robert Walgate

US research overheads

Government semi-retreat

Washington

THE Office of Management and Budget (OMB) bit the bullet last week and published, in the *Federal Register* for 9 June, its final revisions to Circular A-21, the document that determines how institutions are reimbursed for the indirect costs associated with research grants. OMB came under a storm of criticism from universities after publishing its initial proposals for revising Circular A-21 earlier this year.

Indirect costs now represent nearly a third of all payments for research grants, according to OMB figures. To control this steady increase, the new revisions set a fixed allowance of 3 per cent of total research costs for the salaries of department heads and faculty attributable to the administrative overhead costs of research. Institutions receiving federal funds will

not have to fill in forms to receive the allowance, which will eliminate the need for the much-hated "effort reports" formerly required to justify payments for time spent on administrative activities.

According to Milton Goldberg of the Council on Governmental Relations, this

	Deans, department chairs, lab directors	Faculty
Yale University	0.88	5.88
Boston University	5.74	4.82
University of California, Berkeley	0.30	1.54
Harvard University	1.77	2.30

will give a windfall to universities whose costs have been less than 3 per cent. OMB estimates these costs now run between 5.5 and 6 per cent nationwide (see table).

OMB's first shot at a revision of A-21, first published in the *Federal Register* on 12 February, brought a storm of disapproval from universities and their allies in Congress. The proposal would have capped reimbursements for all administrative costs at 26 per cent for the current fiscal year, a figure that was to drop to 20 per cent for 1987. OMB estimated the government would save \$200 million if the original plans had been put into effect. The new plan will save the government only \$100 million.

A principal complaint of universities at the time of the initial OMB proposal was that they had not been consulted. Although OMB held numerous meetings with interested parties in the months following publication of the proposal, Goldberg says its final proposal still came as a surprise. Carol Scheman of the American Association of Universities is encouraged that OMB is taking universities' concern seriously, but is unconvinced the new revisions will solve the problem of rising indirect costs. She maintains that such costs will continue to rise as more money is needed for facilities and equipment.

The new regulations must take effect by 1 July 1987, although individual agencies may implement the changes sooner if they wish. The changes will apply only to new federal grants, leaving existing commitments untouched. But a move by Congress may hold up the new rules until October, the beginning of the next fiscal year. Congressman Sidney Yates (Democrat, Illinois) has amended the House version of a supplemental appropriations bill in a manner forbidding government agencies from spending money to implement the new plan.

The original Senate version of the appropriations bill contained a similar form of words. A House/Senate conference will make a decision this week whether to include the provision in the final version of the bill.

Joseph Palca

Test-tube babies

Tokyo

JAPAN may be on the edge of a test-tube baby boom, according to predictions made by Dr Bob Seamark, an Australian expert in *in vitro* fertilization (IVF), and Dr Masakuni Suzuki, Japan's pioneer of the test-tube baby. Suzuki is so confident of the future that he has built a private hospital near Sendai to deal primarily with infertility problems using IVF techniques. But there are many problems still holding back development of IVF in Japan.

In Japan and Australia the incidence of infertility is similar, with as many as 10–12 per cent of couples unable to produce children. But the number of test-tube babies in Japan stands at only 27 compared with over 1,300 in Australia. Why has Japan lagged so far behind?

The Japanese public and academics are often severe critics of major new medical procedures, as shown, for example, by the outcry against heart transplants and sex selection. But probably more important than this is the lack of government support for IVF research and the cost of IVF treatment. Unlike Australia, IVF is not covered under national health insurance in Japan.

Another major difference between Australia and Japan is the almost complete lack of communication between Japanese scientists involved in reproductive biology research and the clinicians in hospitals who apply IVF techniques. Co-operation between clinicians and scientists, particularly those involved in animal breeding, is, according to Seamark, one of the major driving forces behind the tremendous surge in IVF research in Australia.

David Swinbanks

Japanese gynaecology

Gender selection sparks row

Tokyo

A GROUP at Keio University in Tokyo has caused a sensation by claiming a major medical scoop on national television last month, when it announced that six married women had given birth to baby girls after artificial insemination with X-chromosome sperms separated by centrifugation. But the publicity has back-fired, and the Keio team has come under a barrage of criticism even from within their own university for failing to inform the public of the experiments, and for going ahead without first discussing the ethical questions raised by sex selection.

The ethical committee of Keio University met last week and decided that once the reliability of the method has been established, it should be used only to prevent hereditary diseases. In the meantime, practitioners around Japan have already started using the technique.

Soon after the Keio group made its announcement, a group promoting sex selection headed by Dr Shiro Sugiyama, owner of Sugiyama Gynecology Clinic in Tokyo, revealed that 40 girls had been born around the nation using the Keio technique, and out of 24 women who conceived, 22 gave birth to girls, a success rate of over 90 per cent.

The principle of the technique is simple enough and well known — X-chromosome sperms are marginally more dense than those with Y chromosomes because of the larger size of the former chromosome, and they can, in theory, be separated by centrifugation. Although many claims of successful sex selection based on this principle have been made, no reliable technique has yet emerged. The Keio team, headed by Professor Rihachi Iizuka of Keio University and Professor Hideo Mori of Tokyo University, described their method in 1984 (*Biomedical Research*, 5, 187; 1984).

The secret lies in the fluid in which the sperm are suspended for centrifugation. A density gradient of Percoll, a modified silica gel, is used and the sperm are separated on the basis of differential velocity sedimentation. After a 20–30-minute spin in a centrifuge at 250g, 95–100 per cent of the sperm in the bottom layer carries the X chromosome, while the overlying layer has about 85 per cent sperm containing the Y chromosome. According to Sugiyama, it is then just a simple matter of pricking a hole in the bottom of the centrifuge tube and draining a few drops of the X-chromosome-rich sperm which are used for impregnation.

First treatment in the Sugiyama Clinic costs 50,000 yen (about £170) and, in the case of failure, each subsequent treatment is 20,000 yen. Four or five attempts are

usually required for success.

Sugiyama heads the Sex Selection Research Group which was established 7 years ago and which has 770 practising gynaecologist members throughout the nation. In the past they used vinegar-rinsing of the vagina as a technique to promote the conception of girls. But success was “less than 80 per cent”.

According to Sugiyama, the new method has been used in most cases to prevent hereditary diseases such as haemophilia which occur in men, but he admitted some parents wanted a girl for personal reasons, such as wanting to raise their child to be a ballerina. It is revelations like this that have raised a furore.

Iizuka also initially claimed that the technique had been applied to avoid hereditary diseases, but many Japanese colleagues questioned this and Professor Yasuo Uemura, head of Keio University ethics committee, criticized the Keio team for applying the technique to couples who simply wanted a girl because they already had boys.

Uemura also thought it “regrettable” that Iizuka went ahead without consulting the ethics committee in advance and “imprudent” that some of the members of his team attended meetings of obstetrical practitioners to teach them the sex-selection method.

Immediately after the meeting of the

Keio University ethics committee, Sugiyama said he would abide by the committee's decision and limit application of the technique to prevention of hereditary diseases.

But in a complete about-turn a few days later he declared that he would continue to help ordinary parents to select the sex of their babies until advised otherwise by nationwide medical organizations, such as the Japan Medical Association or the Japan Society of Obstetrics and Gynecology. His change of heart came because he was deluged with letters and telephone calls from people asking him to continue the technique, and he felt it was “his duty as medical practitioner” to respect the wishes of such people.

According to a report in *Shukan Bunshun*, a Japanese weekly publication, the Keio group started clinical trials of the technique in May last year, and for the past six months Japan's national broadcasting corporation, Nihon Hoso Kyokai (NHK), has been allowed to cover the research.

But a difference of opinion arose between the Keio group and NHK about when to release the news; NHK felt that the preliminary success of the technique should be discussed in public while the Keio team wanted to test about 100 women to obtain statistically reliable results.

In the end, NHK was allowed to broadcast after the successful birth of six girls by the Keio technique. **David Swinbanks**

Brown eggs catch on in Japan

Tokyo

FIRST it was the Australian “superpig” and now comes Japan's “biochicken”, the next contender in the farmyard biotech race. And what makes biochicken special? She lays brown eggs, lots of them.

That may not sound like much of a feat until one realizes that shiny reddish brown eggs (literally “red eggs” in Japanese) command twice the price of the paler white variety on the Japanese domestic market. The inflated price of brown eggs in Japan can be attributed to limited supply and a belief among the general public that brown eggs are more nutritious.

Quick to spot a potential money-maker, scientists at Nagoya University and the Agricultural Experiment Station of Aichi prefecture have created White Leghorn hens that lay brown eggs instead of their usual white ones. This is achieved by false fertilization.

Sperm of a Rhode Island Red rooster, which carries the gene for brown eggs, is first irradiated with gamma radiation to inactivate it. The sperm is then allowed to enter the egg of a White Leghorn, thereby introducing some Rhode Island

Red genetic material into the egg which is then fertilized with normal White Leghorn sperm. About 7 per cent of the fertilized eggs result in chickens that lay brown eggs. This technique was developed in New Zealand in 1982 by Pandey, but it had no commercial applications there — in New Zealand brown eggs cost the same as white.

But why go to all this trouble when Rhode Island Reds already lay brown eggs? The answer lies in greater productivity — the White Leghorn is a profuse egg layer. No doubt also, the label “bioeggs” will have commercial appeal in this country where “biolipstick” has swamped the cosmetic market (see *Nature* 314, 395; 1985).

Biochicken, however, is not yet quite living up to expectations; her eggs are only pale brown and are more typical of a hybrid White Leghorn/Rhode Island Red than a true Rhode Island Red.

But by selective breeding the Japanese team is confident that by this autumn it can come up with a hen that lays pure brown eggs, and it has already named this chicken of the future the “Aichi Bio Red”.

David Swinbanks

Colombian immunology

More plans for malaria vaccine

Bogotá

COLOMBIA, hitherto best known for its coffee, now seeks to cultivate a reputation in science. As his last act in support of science, outgoing President Belisario Betancur earlier this month announced a



scheme whereby six of Colombia's best science graduates at a time would be awarded fellowships to study at the Research Institute of Scripps Clinic in California.

As the students will be paid from Colombia, they will be obliged to return there after their three-year stints in Richard Lerner's department of molecular biology at Scripps.

The hope is to increase Colombia's abilities in modern biomedical research, now concentrated in Manuel Patarroyo's Institute of Immunology at the San Juan de Dios Hospital in Bogotá.

Patarroyo, with the aid of the presidency and particularly of one of its economic advisers, Diego Pizano, whose support of science stems from a friendship with French virologist Gerard Bricogne while both were studying at the University

of Cambridge, together with a gift of several sequencing and synthesizing machines from Arnold Beckman, has set up what is probably the best-equipped biomedical laboratory in South America.

Patarroyo, together with approximately thirty enthusiastic young researchers, is beginning to pursue gene cloning and vaccine development for malaria, leprosy and tuberculosis. They do so against the odds. For example, customs and other delays tend to allow "hot" radioisotopes to become cold and heat-degradable enzymes to warm up before they reach the institute. And there is no adequate postgraduate research programme in Colombia.

On the other hand, Colombians do have the advantage over laboratories in North America and Europe of having easy access to malaria and leprosy patients and to the animal models for the diseases, the *Aotus* monkey and the armadillo, respectively.

The most important outcome of the institute's research so far is the development of a candidate malaria vaccine. Reporting the initial data on the vaccine at a conference entitled Perspectives for Chemistry in Medicine in Bogotá this month, Patarroyo described how the immunization of monkeys with a combination of three synthetic peptides, corresponding to partial sequences of one of the major proteins on the surface of malarial red blood cells, protects the animals against infection with the protozoan *Plasmodium falciparum*, which is the main malaria parasite of humans.

So far the tests have involved only a few monkeys, but up to 50 will be included in an extended trial.

About a thousand scientists and students attended the conference. According to the organizers, Patarroyo and Pizano, the main purpose was to provide a stimulus to Colombian science, which came in the form of 10 speakers from the United States and 4 from Europe.

Four of the speakers, Lerner plus Gobind Khorana of Massachusetts Institute of Technology, David Baltimore of the Whitehead Institute and Peter Perlmann of the University of Stockholm, were awarded the Order of San Carlos in the rank of knight by President Betancur during the conference.

Should an effective malaria vaccine emerge — and even with the first phase of clinical trials of some possible vaccine components about to begin in other countries, nobody doubts that there is a long way to go — Colombia should be well placed to carry out a mass vaccination campaign.

During 1985 and early 1986, several million young children were vaccinated

against five common diseases by means of a campaign that involved the newspaper *El Tiempo*, the radio network *Caracol*, UNICEF, the Roman Catholic Church, the armed forces, various government agencies and a host of volunteers.

The only part of the country to be missed out, a strip of inaccessible Pacific coastland, is about to be tackled in a week of action involving 100 helicopters and hundreds of boats.

Peter Newmark

Coffee benefaction

As coffee is far and away Colombia's main export, it is not surprising that the *Federación Nacional de Cafeteros de Colombia* maintains two laboratories, one that concentrates on the chemistry and the other on the biology of coffee.

The chemistry laboratory in Bogotá under Marco Quijano employs approximately twenty scientists and seventy students to study coffee composition and processing as well as tissue culture and the problems of coffee rust, a fungal disease that reached Colombia only in the past two or three years.

Copper-containing sprays are the main way to defeat the rust fungus but they are expensive to administer, not least because they have to be delivered by hand as aerial spraying would ruin the electricity cables. The chemistry laboratory is conducting a careful study of the effects of copper on the coffee plant, to establish whether it would be possible to treat the soil with copper to inhibit rust disease. In the end, however, Quijano believes that the fungus will develop resistance to copper.

Studies of coffee processing are a particular focus of the laboratory because of the growing demand for decaffeinated coffee in the developed nations. There has been talk of using biotechnology genetically to manipulate the coffee plant so that the beans are caffeine-free. But in economic terms, this makes little sense, because the process of decaffeination is paid for by the sale of the caffeine to the pharmaceutical industry. Cloning of coffee plants, on the other hand, is considered important and 250 plants derived by tissue culture are being studied.

Colombian coffee tends to be sold at a premium price because it is made from ripe beans only. That is possible because the beans are hand-picked rather than mechanically shaken from the plants. This fits with the fact that Colombian coffee is largely grown on small plots owned by a single family which receives a guaranteed price from the coffee growers' association to whom they have to sell their crop. This provides a buffer against bad years, but also means that the farmers are not benefiting from this year's high prices, occasioned by the failure of the Brazilian crop.

Catch your monkey

AOTUS monkeys are collected by Indians in the Amazonian basin. With a group of monkeys inside the hollow tree trunks that they live in, the Indians first seal off all the exit holes. They then reopen one hole and as the enraged animals emerge, try to catch them by the tail.

It can take a week to catch a single animal. Captured monkeys are taken to Leticia at the southernmost tip of Colombia, where the malaria trials are carried out. Because the *Aotus* monkey is a protected species, those that succumb to malaria have to be cured with drugs. They are then passed on to a breeding colony elsewhere in Colombia and, sometimes, released back into the wild. □

The Paluxy River mystery

SIR—The article "On the Tracks of Men and Money" by Tony Thulborn (*Nature* 320, 308; 1986) contains many inaccuracies on a subject that cries for clarity. The presumed occurrence of both human and dinosaur tracks in the Lower Cretaceous Glen Rose limestone in central Texas has for over a decade been the favourite illustration of the creation model, while being, as Thulborn personally admitted to me, "the evidence evolutionists feared the most".

It is true, as Thulborn stated, that, at a January 1986 lecture at the University of New South Wales, I called attention to recent changes in the nature of the tracks which bring into question the original interpretations by myself and others. Having been involved in this project for a decade and having written the definitive book on the subject, I did not find this comfortable, but as an empiricist and honest man, I dared not do less. Even though Thulborn calls the present nature of the tracks "embarrassingly obvious" and the process by which they changed "well known", the truth is, evidently, that an unprecedented geological phenomenon has taken place which is, as yet, only poorly studied and less well understood.

Somehow, since 1984, surface stains in the shape of tridactyl dinosaur prints have appeared, surrounding many of the eroded remnants of elongated tracks once interpreted as human-like. In almost all cases, these stains are not associated with any impression in the rock — they are a coloration phenomenon only. In some cases, the stain ingores the "mud up-push" surrounding the human-like impressions.

Neither my two field trips to the Paluxy in the fall of 1985, nor the analysis of samples taken, answer all the questions. There are only two possibilities: (1) an, as yet, undeciphered geological process partially obscured portions of the tracks of a previously unknown dinosaur which walked in an atypical plantigrade fashion, or (2) that someone has fraudulently altered the appearance of the tracks.

I emphasize that I have seen no direct evidence of fraud. I plan to continue my research once water levels are lower, to gain a more complete understanding, but meanwhile I have encouraged creationists to refrain from using the Paluxy as an anti-evolutionary argument.

Thulborn and other evolutionists are rushing to embrace this evidence, relishing the fact that the tracks, once thought to be human, now appear reptilian. But hitherto, the usual evolutionists' explanation has been that the markings were not tracks at all. The 1986 booklet *Creationism, an Australian Perspective* by the Australian Skeptic Society implied that I had carved some of them. Others have

claimed they were scour marks, eroded potholes, worm burrows or creationist footprints in the river-bottom mud. After studying both the present nature of the prints and photographs of their nature when first discovered, I am convinced that the original interpretation was not only valid, but arguably the best.

Thulborn, president and founder of the Australian Association for the Protection of Evolution (APE), is also less than objective when he repeats the Skeptics' charge of financial impropriety on the part of the Creation Science Foundation (CSF) of Australia. He claims "CSF still declines to give a detailed explanation" of the whereabouts of \$92,000. However, he should know (I was standing next to him when the president of CSF explained it to him) that an employee of the investment company in which the money had been placed had embezzled from many accounts, including that of CSF, and that the authorities had frozen the remaining assets. Court cases are now in progress. So far CSF has received only a portion of its money back.

Likewise, Thulborn repeats the charge that federal money had wrongly been given to CSF in the form of an "export market development grant". My understanding is that the Australian government financially encourages companies and organizations that export products to be sold overseas; CSF has recently begun to publish and print locally a magazine and other publications which are primarily sold in North America and Europe and, as such, is eligible for such an export development grant.

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SIR—Tony Thulborn reports (*Nature* 320; 308; 1986) that Robert Morris now admits that the "human" footprints on those of big dinosaurs in Cretaceous limestones, Paluxy River, Texas, were those of small three-toed dinosaurs. Additional news is now available from San Diego, California, that the fake exhibit at the Institute for Creation Research, showing an 80-million-year-old human being beside a dinosaur¹, has been "closed for revision", and that the "casts" have been removed. Will there be a revision of *Dry Bones*²?

There is more joy, among evolutionists, over one sinner that repenteth than over ninety and nine that are too brazen to repent. Can we hope that other creationist intellectual atrocities may be abjured by their perpetrators? Examples are misrepresentations of the second law of thermodynamics and of isotopic dating, claims for variability of the speed of light, and

engagement, by good and bad angels, in their own form of star wars³.

Many evolutionists will be glad to help the creationists put their house in order, even at the expense of its disappearance into thin air.

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1. Jukes, T.H. *Nature* 308, 390–400 (1984).

Cometary first

SIR—The magnificent 15 May 1986 issue of *Nature* containing the first results from the spacecraft voyages to comet Halley was marred by an unfortunate lapse in the Editorial (p. 366) by John Maddox entitled "First journey to a comet". For the record, the first encounter took place on 11 September 1985 when the International Cometary Explorer passed 7,800 km tailward of the nucleus of comet Giacobini-Zinner. Of the missions to comet Halley, only Giotto came closer to the nucleus.

The field of cometary research has been advanced in 1985 and 1986 by a massive effort from a variety of disciplines. We applaud the work of the Giotto, Sakigake, Suisei and Vega projects for their pivotal contribution. However, appreciation of this achievement does not require a revision of history.

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Safe nuclear energy

SIR—The important lesson from the Chernobyl disaster is that the best brains in the world are needed urgently to work on developing safe nuclear energy, and that the warnings of these scientists must be heeded even when they say things that their governments and the nuclear power industry do not like. Unfortunately, one of the best brains in the nuclear safety business has been out of the action for several years. Andrei Sakharov has been isolated, harassed and prevented from contributing to this important effort because he says things that his government does not like.

If General Secretary Gorbachev is really serious about the need for a collective world effort to make nuclear energy safe, he should bring this great pioneer of nuclear safety back into the mainstream of nuclear research.

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When reference means deference

Hoyle and Wickramasinghe protest that none of the contributors to recent accounts of the encounters with Halley's comet have referred to them. But they have only themselves to blame.

WHAT follows is a sad but disturbing tale, which is told not for the sake of making further trouble but in the hope of stilling what there is already. It centres on the recent encounters of the two Soviet Vega spacecraft and the European Giotto spacecraft with Halley's comet in March, the publication of preliminary accounts of the encounters in *Nature* on 15 May, the publication in the succeeding issue (321, 385; 1986) of a "prediction" (published after the event) by Professor J.M. Greenburg at the University of Leiden that the surface of the nucleus of Halley's comet would appear black and the complaints of two British scientists, Sir Fred Hoyle and Professor Chandra Wickramasinghe, that their own earlier predictions to the same effect have wilfully and even maliciously been overlooked.

Most readers will not have ready access to the documents in which the complaints surface, which consist chiefly of "pre-prints" circulated from the Department of Astronomy at University College, Cardiff. The description of this now considerable volume of largely unpublished literature is a misnomer, because much of it is plainly not written with the intention that it should go through the processes that ordinarily precede publication, scrutiny by referees, for example. This is certainly the case with "Cardiff Astrophysics and Relativity preprint No. 125", dated 1 June 1986, under the title *On deliberate mis-referencing as a tool of science policy*, which has been artfully contrived to appear as if it were the galley proof of a letter due to appear in *Nature*. (For future reference, the authors of this little joke should know that our type size is known in the trade as "9 on 10", not "9 on 11", and that our wide columns are 20.6, not 20.0 "ems" wide.)

Readers will also know that Sir Fred Hoyle is one of the most talented and ingenious theoretical scientists since the Second World War. With Bondi and Gold, he launched the steady-state theory of cosmology, with a variety of collaborators (W.H. Fowler and the two Burbidges) he laid down the outlines of the theory of nucleogenesis and, almost as if in passing, showed how to calculate the evolution of stars. Latterly, Hoyle has been exploring a question originally posed in urgent form by Wickramasinghe, that of the constitution of interstellar dust. Many readers will know the eccentric direction this work has taken. Hoyle and Wickramasinghe

have remarked that carbon is a common constituent of interstellar dust (as it is of gas), that much of this carbon is in the form of sedimentary organic molecules and have then gone on, in a series of mostly *samizdat* publications, to argue that the prevalence of interstellar carbon only goes to support one of the other theories to which they are attached. They say that life as it is known on the surface of the Earth is genetically far too complicated to have arisen by means of darwinian evolution from primordial chemicals, that at least the first steps in the evolution of life are likely to have taken place "out there" and that, as it happens, comets are likely to have been among the means by which the surface of the Earth is from time to time repopulated by external organisms.

Those writing off for a copy of preprint 125 should be sure also to ask for preprint 121, dated 1 March, which put forward a particular model of the constitution of the periodic comets (of which Halley is one). Most of the objects, the argument goes, will have lost volatile material from the outer surface, which will therefore be a loose uncompacted "protective" skin of material with low refractive index and, therefore, low reflectivity for sunlight. The authors are partly concerned to explain the discrepancy between the calculated and observed (only a tenth as many) number of periodic comets; their solution is to suggest that most periodic comets slip by unobserved, but that those with more than one nucleus become visible because the nuclei rub against each other, exposing parts of the volatile interior to solar radiation. Hoyle and Wickramasinghe did not on 1 March say so explicitly, but it may be inferred from their general discussion that the surface of Halley's comet was predicted to appear mostly black.

The complaints in preprint 125 are more entertaining. First, they marvel at Greenberg's "sibylline achievements" in predicting what Halley's comet would seem like after the event, overlooking that Greenberg's model is radically different from their own; in fact the reason for its publication was not that it made a "prediction" but that it is a model of which many readers of *Nature* would not previously have heard. Second, they remark, in sorrow rather than anger, that none of the Halley authors in the issue of 15 May refers to them. They go on to note that, in its time, *Nature* has been guilty of removing a reference to their work from an article writ-

ten by a third party. Finally, they darkly speculate on the possibility that this neglect may simply have been a consequence of this journal's malign attitude both towards themselves and as a willing "vehicle of propaganda for Darwin's theory". They suspect that it may be a "management condition" that "persons judged to be a threat to Darwinism are to be blotted out from the scientific world".

This lengthy reply to what Hoyle and Wickramasinghe allege stems not so much from the circumstance, attested by preprint 125, that the authors and the present editor of *Nature* are old friends but from a wish that these two talented people should come to see the error of their ways. But in passing, it should be known that the sinister deleted reference was a reference to their panspermiology in D.A. Allen and D.T. Wickramasinghe (*Nature* 294, 239; 1981), an otherwise helpful measurement of infrared absorption in interstellar space.

Moreover, they may be right about comets. Much of what they have written over the years deserves to be widely read, for example the argument that comets contain polyformaldehyde (Vanysek, V. and Wickramasinghe, N.C. *Astr. Space Sci.* 33, 19; 1975) or the demonstration (with W.K. Wallis) that comets Cernis and Bowell probably contain organic molecules rather than water-ice (*Earth, Moon and Planets* 33, 179; 1985). What these authors seem incapable of understanding is that their panspermian convictions sully even their sober contributions to the literature. People who might (and even should) read their articles on comets do not do so for the simple reason that they half expect to find there a rehearsal of the now familiar and widely popularized view that life began in space.

Not that even that is necessarily wrong. Time may yet show that Hoyle and Wickramasinghe have been true prophets crying in the wilderness of disbelief. Yet as things are, there is no evidence but conjecture on their side. Their flimsy account of the origin of life has as its sole foundation their scepticism of the efficacy of natural selection among primordial molecules. It is as if a particle theorist chose to say that, while the Higgs boson remains undiscovered, the only partly successful theory so far must be discarded and all others, however flimsy, must be given more than equal time. They ask a lot, too much for most.

John Maddox

Endocrinology

Dual gonadal control of follicle-stimulating hormone

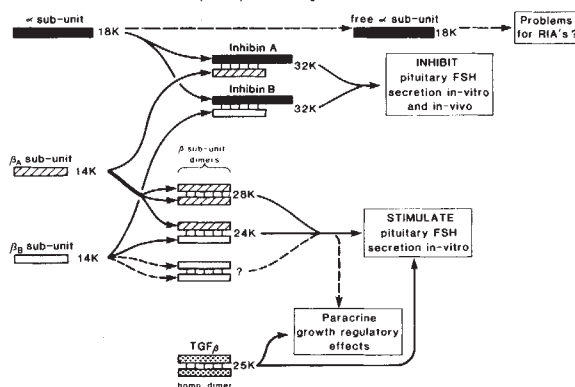
from Con G. Tsonis and Richard M. Sharpe

OF all the reproductive hormones, none has had a more chequered history than inhibin, a glycoprotein produced by the gonads that feeds back to the anterior pituitary gland to decrease specifically the secretion of follicle-stimulating hormone (FSH)¹. Attempts at the purification of inhibin proved far from straightforward. Reports of molecules with inhibin-like activity ranging in relative molecular mass (M_r) from 10,000 to 100,000, (10K–100K) raised doubts about the authenticity of inhibin. These have recently been resolved by demonstration that is composed of an interlinked α - and β -subunit. Now comes a major surprise. Two reports on page 776 and 779 of this issue^{2,3} show that dimers of the β -subunits of inhibin are potent stimulators of FSH synthesis and secretion, raising the possibility of dual control of FSH from within the gonads.

Clarification of the nature of inhibin occurred in two phases. In 1984 a low M_r (14K) form of inhibin, present in human seminal plasma, was purified and sequenced⁴, but was later shown to originate from the prostate and not the gonads⁵. It is now known to be structurally unrelated to gonadal inhibin. This probably excludes it as a physiological regulator of pituitary FSH secretion. More importantly, in December 1985 the full sequence of two 32K forms of porcine follicular fluid (pFF) inhibin (inhibin_A and inhibin_B) were published⁶, and have since been confirmed for bovine inhibin⁷. Each form is composed of two crosslinked subunits (see figure), a larger common α -subunit of M_r 18K and a smaller, distinctive β -subunit of M_r 14K, designated as β_A or β_B according to differences in the amino terminus. As these were the predominant forms of inhibin present in pFF and, as higher molecular mass forms of FF inhibin (for example, 58–108K) can probably be explained by extensions of the α - or β -subunits^{7,8}, it appeared that inhibin had finally yielded its main secrets.

Two independent laboratories now report the isolation and purification of two inhibin-related factors from pFF which are potent and selective stimulators of pituitary FSH release *in vitro*. These factors are referred to as FRP (ref. 2) and activin³, respectively, by the authors. FRP is a 28K polypeptide composed of two β_A subunits of inhibin and activin is a poly-

peptide composed of one β_A and one β_B subunit of inhibin held together by disulphide bond(s) (see figure). Although only two β dimers were isolated this does not exclude the possibility of a third factor, a homodimer composed of two β_B subunits. For the β dimers to have biological activity both subunits must be joined together because reduction of the disulphide bonds destroys their bioactivity, as is also the case for inhibin. The β dimers are highly potent stimulators of both the biosynthesis and secretion of FSH but have no effect on luteinizing hormone (LH) or prolactin. The β_A



Diagrammatic representation of how the α - and β -subunits of porcine follicular fluid inhibin can be combined to yield molecules with either FSH-inhibiting or FSH-stimulating activity. —→ Proven pathways; - - - possible pathways. Reported relative molecular masses are shown.

homodimer is reported also to reduce the secretion of growth hormone and adrenocorticotrophic hormone².

The action of the β dimers can be distinguished from those of luteinizing hormone-releasing hormone (LHRH) in several ways. Compared with LHRH, β dimers have no effect on LH secretion, act much more slowly (greater than 4 hours rather than minutes for LHRH) and have greater stimulatory effects on the biosynthesis of FSH. Also, LHRH antagonists, which completely block the LHRH-stimulated release of both FSH and LH *in vitro*, have no effect on the secretion of FSH mediated by the β dimers. These data suggest that the β dimers act through receptors separate from those for LHRH and probably via a different mechanism.

An additional finding of great interest is the striking homology between the β dimers and transforming growth factor- β (TGF- β)⁶; the homology is such that they are considered to be products of the same gene family. TGF- β exists as two 12.5K sub-units linked by disulphide bond(s) to

yield a 25K homodimer (see figure) which is also a potent stimulator of FSH release at low concentrations (10^{-10} M)⁹.

The discovery that the β dimers are present in follicular fluid together with inhibin suggests that there is a 'push-pull' system operating from within the gonad to control production of FSH. When both inhibin and the β dimers are added to pituitary cells, the effect of inhibin predominates and FSH secretion is decreased. As inhibin is present in follicular fluid in higher amounts than the β dimers this implies that negative control of FSH by the gonad is the principal feedback message, though this does not exclude the possibility that secretion of the β dimers may predominate under different physiological states.

However, before critical evaluation of the role(s) of the β dimers is possible it will be necessary to establish first, whether they are secreted from the gonads into the peripheral circulation; and, second, whether they have local growth effects within the gonads analogous to the effects of TGF- β reported in other tissues¹⁰. If the β dimers are secreted systemically then problems would arise if they also had TGF- β -like activity because the latter has widespread local effects in various tissues and the β dimers might activate these sites. The structural differences between the β dimers and TGF- β may prevent this.

Alternatively, the β dimers may not be released systemically but the ability of the β dimers (or TGF- β) to increase pituitary FSH release at picomolar concentrations or less *in vitro* suggests strongly that this effect is of some physiological importance.

Indeed, if the β dimers are not secreted from the gonads into the blood, then perhaps a molecule resembling the β dimers or TGF- β is secreted by the hypothalamus to modulate pituitary FSH. There are several reports of the existence of a hypothalamic FSH-releasing hormone¹¹, but these have never been widely accepted and appear to describe a smaller molecule than the β dimers. With respect to the possible local effects of the β dimers, it has been shown recently that TGF- β can enhance FSH-stimulated secretion of oestradiol by rat granulosa cells *in vitro*¹². This suggests that the β dimers may normally exert a comparable effect within the ovarian follicle. In the same study, 32K inhibin was shown to antagonize FSH-stimulated secretion of oestradiol, raising the possibility of dual local modulation of FSH action within the gonad.

These recent developments have important implications with regard to the measurement of inhibin by radioimmunoassay¹³ and bioassay¹. The relative abun-

dance in the ovary of the messenger RNAs for the α -subunit precursor of inhibin is estimated from Northern blots to be at least 10-fold greater than that for the β_A precursor, and about 20-fold greater than for the β_B precursor⁶. This suggests that there may be secretion of free α -subunit or its precursor. As free α - or β -subunits are devoid of FSH-releasing or inhibiting activity, antibodies directed towards the α -subunit of inhibin may cross-react with the free α -subunit, therefore overestimating the amount of inhibin in a sample. In a preliminary report⁸, however, free α -subunit of the hormone could not be detected in bovine follicular fluid.

Antibodies raised against the β -subunits of inhibin (β_A or β_B) may prove even more troublesome in that they may well cross-react with the β dimers as well as with inhibin. This again would lead to an overestimation of the levels of inhibin. Similar problems can be expected with antibodies raised against the β dimers, as these may show major cross-reaction with inhibin. Furthermore, bioassays of inhibin may underestimate the actual level of the hormone in a sample if the β dimers are also present and countering the effects of inhibin.

Until now it has been an accepted principle of endocrinology that gonadal feedback control of FSH secretion is under the dual control of steroids and inhibin. Intro-

duction of β dimers or an equivalent as a third candidate would necessitate a major rethink about control of the hypothalamic-pituitary-gonadal axis. Finding out how the function of this third factor interacts at the pituitary level with the actions of LHRH and with those of the recently described LHRH-associated peptide (GAP)^{14,15} and elucidating the local roles of inhibin and the β dimers undoubtedly presents reproductive endocrinologists with a considerable task but it also promises more surprises. □

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Precambrian geology

The most ancient rocks revisited

from Stephen Moorbath

THREE years ago, a group of workers from the Australian National University surprised the geological world by reporting¹ uranium-lead (U-Pb) dates of between 4,100 and 4,200 million years (Myr) for 4 detrital zircons out of 102 analysed from the Mount Narryer quartzite in Western Australia, which was probably deposited and metamorphosed about 3,600–3,350 Myr ago. On page 766 of this issue², W. Compston and R.T. Pidgeon report a further occurrence of such old detrital zircons, identified once again with the ion-microprobe SHRIMP, in a conglomerate from the Jack Hills metasedimentary belt at a site about 60 km north-east of Mount Narryer.

In Compston and Pidgeon's study, 17 zircon grains out of 140 analysed yield U-Pb dates greater than 4,000 Myr. Indeed, one zircon grain registers the exceptionally old date of $4,276 \pm 6$ Myr, which may still be a minimum value for its original age. The authors regard the 'old' zircon population as either having a single original age close to 4,300 Myr with subsequent early as well as recent Pb loss, or as

a mixed-age population that formed during discrete events over an extended time period between 4,100 and 4,300 Myr ago. The age of these ancient minerals constrains the time of the earliest preservation of Earth's solid crust.

As at Mount Narryer, the majority of zircons at Jack Hills register very much younger dates than 4,000 Myr, probably related to igneous and/or metamorphic thermal events 3,100–3,500 Myr ago. In principle, early gain of Pb (or loss of U) followed by recent Pb loss could produce anomalously old apparent U-Pb zircon dates, but although this process has been demonstrated in a zircon from Mount Sones, Antarctica³, it is not regarded by the Australian workers as a plausible model for Mount Narryer and Jack Hills.

What clearly needs to be done next is to subject the Jack Hills zircon population to conventional solid-source mass spectrometry and isotope dilution analysis of single grains and individual fragments of single grains. Schärer and Allègre⁴ performed this type of analysis on 32 single zircon grains from Mount Narryer; they obtained

no 4,100–4,200 Myr dates and interpreted the results solely in terms of zircon crystallization 3,800–3,300 Myr ago. From simple statistics, Compston *et al.*⁵ countered by postulating that Schärer and Allègre had simply been unlucky in failing to find any zircons more than 4,100 Myr old at Mount Narryer. (The chance of selecting 32 'young' grains in succession was shown to be 27 per cent.) Because the proportion of 'old' zircon grains at Jack Hills is about five times that at Mount Narryer, the conventional analytical approach will be greatly facilitated.

The morphology as well as the U and Th contents of the Jack Hills zircons suggest to Compston and Pidgeon that their source consists of a mafic rock which has undergone granulite-facies metamorphism. In contrast, the source rock at Mount Narryer may have been a felsic igneous rock not recrystallized by granulite-facies metamorphism. Thus, the ancient source terrain of the detrital zircons, not yet found *in situ*, may have had considerable geological complexity although, as I pointed out in a previous *News and Views* article⁶, one should be cautious in attributing a truly continental character to the source-region crust. Perhaps the ancient source terrain was more like an early non-continental volcano-sedimentary assemblage of greenstone belt facies, such as the now well-known Isua Belt of West Greenland which, with a well-established age of ~3,800 Myr, is the oldest *in situ* terrestrial rock assemblage known^{7,8} and has survived in a relatively pristine condition until the present day. It is highly significant for any model of early crustal evolution that a 4,100–4,300-Myr-old source region should have remained exposed for at least 600–800 Myr at such a very early period of the Earth's history and preserved from recirculation into the mantle by plate tectonics or by any other process.

It may be too much to hope that the source rocks of the oldest Mount Narryer and Jack Hills zircons will actually be found *in situ* and thus become amenable to dating by other isotope methods. In the meantime, I shall try to repress a recurring nightmare that SHRIMP will one of these days come up with zircon U-Pb ages significantly greater than 4,600 Myr, the widely accepted age of the Earth. It would not be the first dating method to have undergone this embarrassing trial, and yet emerged triumphantly. □

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Archaeology

Finding the earliest Americans

from Warwick Bray

THE problem of the age and nature of the first human colonization of the New World has divided the archaeological profession for more than a century. Even now, there is general agreement on only two points: that the source of the colonizing population was north-east Asia; and that man was around by 11,500 years before present (BP), when fluted stone spear heads (Clovis points) are documented at several localities in North America. The Chilean site of Monte Verde¹ (13,000 yr BP), and perhaps the Alice Boer locality in Brazil² (14,200 $\pm$ 1,150 yr BP), push back the threshold by a few millennia, but no material older than 15,000 yr BP has gained universal acceptance. The strong evidence for stone tools dated at 32,000 yr BP at a site in Brazil, reported by Guidon and Delibrias on page 769 of this issue³, is therefore important to archaeologists and to palaeontologists. The controversial belief that human predation caused the extinction of many large mammals at the end of the Pleistocene (that is, the Clovis point period)⁴ is much weakened if large populations of hunters were already present in the Americas some twenty thousand years earlier.

Recently, the proponents of very early dates have suffered two setbacks. Using a corrected calibration, the apsarctic acid racemization ages of the California 'Palaeo-indian' skeletons have been drastically revised, and all these specimens turn out to be Holocene⁵. Still worse is the revised date for the only undisputed bone tools from the Old Crow Basin in the Yukon. In the 1970s, carbon extracted from the inorganic apatite fraction of a fleshing tool made of caribou rib gave a radiocarbon age of about 27,000 yr BP, but a re-dating of organic proteinaceous material (by the accelerator mass spectrometry method) has reduced this age to 1,350 $\pm$ 150 yr BP, though the dates for the redeposited (and possibly flaked) mammoth bones from these gravels remain more or less the same as the original date⁶.

In essence, however, the basic dispute is not about individual sites or artefacts, but about what constitutes archaeological proof. It is true, of course, that piling up dubious cases proves absolutely nothing, and that a single unassailable find is enough. In theory, the criteria for acceptability are simple: there must be man-made artefacts (or other signs of human presence, such as hearths or structures) datable either directly or by their occurrence in deposits of known age. The ideal site would have a long stratigraphy, plentiful tools, multiple radiocarbon dates and

evidence for evolution within the stone industries. This is exactly what Guidon and Delibrias provide in the work on the Brazilian site of Boqueirao of Pedra Furada³. Their oldest carbon-14 date, 32,160 $\pm$ 160 yr BP, comes from a fire hearth in a stratum with flaked tools and with spalls of painted rock from the walls of the shelter. Another sixteen radiocarbon dates are scattered at intervals through 3.5 metres of sediments. The validity of their evidence seems beyond doubt, and the excavation also suggests that cave art in the New World began at much the same time as in Eurasia and Africa.

It is to be hoped that Pedra Furada does not suffer the neglect given to Tlapacoya, in the Basin of Mexico, where good artefacts were discovered more than 20 years ago in a long sequence of lacustrine sediments and volcanic ashes dated at about 24,000 yr BP⁷. And there are other reports which, if eventually confirmed, will strengthen the argument for early colonization. At El Cedral, Mexico, stone tools and extinct fauna are provisionally dated at more than 30,000 yr BP⁸ and in the deepest strata at Monte Verde, Tom Dillehay (personal communication) has found a split basalt pebble, wood fragments, two modified stones and charcoal dated at about 33,000 yr BP.

Evidence supporting an early colonization is also emerging from other disciplines. A study by Richard Rogers⁹ of linguistic diversification in North America

suggests that man must have been present south of the ice sheets before the Wisconsin maximum of about 18,000 yr BP, and long before the (post-glacial) incursions of the Na-Dene and Eskimo-Aleut language families.

A parallel study of the dental evidence by Christy Turner II¹⁰ also indicates three separate colonizations, equating with the three major language introductions. He proposes that colonists initially spread outwards from North China around 20,000 yr BP and moved, via the Lena Basin of Siberia, into Beringia and Alaska. By the end of the Pleistocene this same, early Amerindian population had got as far as Tierra del Fuego. The descendants of Turner's proposed original group of immigrants could have filled up the Americas and must have been responsible for all stone industries older than 10,000 yr BP, when microblade tools and Na-Dene languages came to Alaska. In the light of the evidence from Pedra Furada we may now have to push Turner's starting date back by more than ten thousand years. □

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Caldesmon

Thin filament regulatory proteins of smooth- and non-muscle cells

from Anthony Bretscher

THE mechanism by which changes in the concentration of cytosolic calcium regulate the contractile machinery of smooth muscle has been a controversial problem. But the situation has now changed dramatically with the emergence of evidence in favour of a Ca²⁺-calmodulin-mediated filament regulatory system.

The now well-established discovery^{1,2} that the interaction of myosin light-chain kinase and Ca²⁺-calmodulin leads to the phosphorylation of myosin and thereby enhances the actin-activated ATPase of myosin was a major step forward. It led to the common belief that smooth muscle contraction is controlled by the regulation

of thick filaments. This mechanism contrasts with that in striated muscle, which is regulated by the classic tropomyosin-troponin thin filament system.

The smooth muscle thick filament regulatory model was extended to non-muscle cells when regulation of some non-muscle myosins via light-chain phosphorylation and dephosphorylation was found³. Overwhelmed by the simple elegance of the phosphorylation mechanism of smooth muscle thick filament regulation, scant attention was paid to suggestions of thin filament regulation — that is, thin filaments that activate myosin in the presence, but not in the absence, of Ca²⁺.

During this period, Ebashi and colleagues¹ suggested that a protein called leiotonin might impart some regulation, although this has not been independently substantiated.

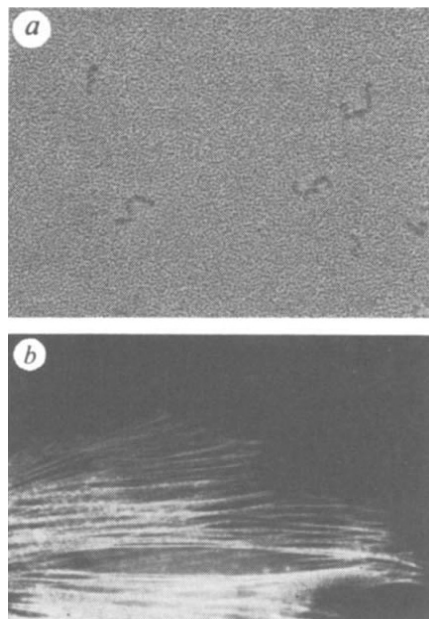
The first evidence that suggested thin filament regulation of smooth muscle contraction was in systems containing activated myosin, but gave no clear indication of the regulatory molecules involved. As these experiments were refined, interest focused on the major components of partially purified thin filaments that retained Ca^{2+} regulation, namely actin, tropomyosin and a polypeptide of high relative molecular mass ($M_r \approx 120,000$ (120 K))². The second line of evidence was the identification in chicken gizzard smooth muscle of a protein that binds both actin filaments (F-actin) and Ca^{2+} -calmodulin³. This protein, with an apparent polypeptide $M_r \approx 140$ K, was named caldesmon (from a combination of calmodulin and $\delta\epsilon\sigma\mu\sigma$ — bond) because its binding to F-actin can be inhibited in the presence of calmodulin in a Ca^{2+} -dependent manner. Further characterization in a reconstituted contractile system (consisting of desensitized actomyosin, myosin light-chain kinase, tropomyosin and limiting calmodulin) revealed that caldesmon inhibits the phosphorylated actin-activated ATPase of myosin⁴. This system can be regulated by the addition of excess Ca^{2+} -calmodulin which relieves the inhibition imposed by caldesmon (refs 4,6, but see ref. 7 for an alternative view). The two lines of evidence merged when it was found that the 120 K thin filament protein is caldesmon⁴. The inhibitory activity of caldesmon can be inactivated by phosphorylation of the protein by a specific protein kinase, which suggests another level of regulation. This caldesmon kinase activity is activated, like myosin light-chain kinase, by Ca^{2+} -calmodulin⁷.

Taken together, these data indicate that smooth muscle is regulated at several levels by Ca^{2+} -calmodulin: first, regulation of myosin by reversible phosphorylation; second, Ca^{2+} -calmodulin binding to caldesmon which relieves the inhibitory effects of caldesmon on the actomyosin interaction; and third, regulation of caldesmon by reversible phosphorylation via the Ca^{2+} -calmodulin-regulated specific kinase and a phosphatase.

So what is known about the molecular properties of caldesmon? The protein appears to be structurally very stable, as it can be boiled in high salt or precipitated with organic solvents, yet retains its characteristic properties. It is a mildly acidic protein that, at least in chicken gizzard, exists as two polypeptide chains of slightly different apparent M_s that can each be resolved into several isoelectric variants⁸. Hydrodynamic data indicate that caldesmon is highly asymmetrical⁹; low-angle rotary shadow electron micro-

graphs confirm this and reveal that it is a long, flexible molecule (*a* in the figure)⁹.

Caldesmon seems to be widely distributed as immunologically related proteins have been found in all smooth and non-muscle cells so far examined (*b* in the figure)^{10,11}. Here the story becomes perplexing. There is a great deal of heterogeneity in caldesmon species in different cells: the apparent M_s of caldesmons from different smooth muscles varies although they are in the region of 140 K (ref. 9). In various non-muscle tissues and cultured cell lines the apparent heterogeneity becomes even more noticeable. Some tissues and all the cell lines so far examined contain two classes of immunoreactive polypeptides: those in the M_r range 120–150 K



a, Low-angle rotary shadow electron micrograph of smooth muscle caldesmon ($\times 43,000$). *b*, Indirect immunofluorescence micrograph of part of a cultured A7r5 smooth muscle cell showing localization of caldesmon ($\times 530$).

and those in the range 71–77 K¹¹. Because these species all remain soluble after heat treatment and bind F-actin in a Ca^{2+} -calmodulin inhibitable manner^{11,12}, they are functionally related to smooth muscle caldesmon, but nothing is known about how they are related to one another. Caldesmon is present along stress fibres of cultured fibroblasts in a periodic pattern apparently coincident with the localization of myosin, myosin light-chain kinase and tropomyosin, and complementary to the distribution of α -actinin¹¹. In smooth muscle, caldesmon is localized in the actomyosin domain rather than in the apparently non-contractile filamin domain⁹. Thus it is not only capable of regulating the actomyosin interaction *in vitro*, but is also localized in the correct region of the contractile apparatus of smooth and non-muscle cells to perform this function *in vivo*.

How does caldesmon regulate the acto-

myosin interaction? Is it, together with tropomyosin, a primitive form of the tropomyosin-troponin regulatory system of striated muscle? We do not yet have enough information to answer either of these questions. But any model must take into account the following observations. First, the apparent regulatory properties of caldesmon seem to require the presence of tropomyosin^{4,6}. The co-localization of caldesmon and tropomyosin along stress fibres of cultured cells may also indicate a regulatory role for tropomyosin. Tropomyosin has been suggested to stabilize selectively certain classes of microfilaments. In the light of the results I have described here, it seems more likely that caldesmon and tropomyosin are part of a contractile unit that regulates actomyosin interaction.

Second, the molar ratio of caldesmon to actin is surprisingly low for a putative regulatory protein, far below the level of tropomyosin. How can a protein with low stoichiometry to F-actin regulate the actomyosin interaction? Could caldesmon alter the interaction between tropomyosin and F-actin over considerable distances and thereby regulate the interaction? Or is caldesmon perhaps selectively associated with restricted domains of F-actin-tropomyosin filaments involved in contraction? Finally, the ability of Ca^{2+} -calmodulin to inhibit the binding of caldesmon to F-actin requires a large molar excess of calmodulin over caldesmon^{6,8}, a level in excess of that reported for chicken gizzard. Perhaps that's just life in a test-tube under the wrong conditions, or missing another component. An alternative explanation may be that caldesmon release from F-actin is not required for relief of the actomyosin inhibition⁴. To explore these questions further, we need to know more about the interaction of caldesmon with calmodulin, F-actin and other (identified or yet to be identified) components. In addition to these questions, the possibility that caldesmon is a 'latch' between the thick and thin filaments needs to be examined. □

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Cell biology

The fibronectin receptor family

from Maria Leptin

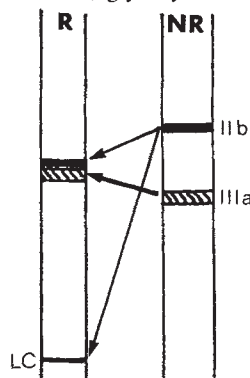
IN a developing embryo cells and tissues are constantly on the move. Individual cells migrate over long distances and sheets of cells rearrange during processes such as gastrulation and neural tube formation. During wound healing and metastasis in cancer similar situations are re-created in adult organisms. The extracellular matrix (ECM) provides a guiding substrate for cell movement, and, *in vitro*, its components can serve as an attachment substrate for cells to spread and migrate on plastic surfaces. How do cells bind to the ECM and what is the link between the ECM and the cytoskeletal motor? Do different cells use the ECM to migrate to different places and, if so, how? The properties of a family of recently isolated receptors for fibronectin and other ECM components suggest that a complex system of receptor-ligand interactions governs cell movements.

Two paths led to the identification of fibronectin receptors. One used the cell binding domain of fibronectin to purify proteins from cell lysates by affinity purification. The success of this approach depends on a specific elution of material bound to the column. An obvious candidate for an eluant is the hexapeptide Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) containing the recognition sequence RGD of the fibronectin cell-recognition domain, especially as the peptide inhibits the binding of cells to fibronectin substrates, and of soluble fibronectin to cells. The second approach was to immunoprecipitate cell surface proteins with antibodies that inhibit cells from binding to fibronectin.

With these two methods similar cell surface proteins, each consisting of at least two subunits, were purified from a diversity of cell types¹⁻⁷ including epithelial, erythroid and myogenic cells and thrombin-activated platelets. The subunits of the platelet proteins are the previously identified glycoproteins IIb and IIIa. The RGD-containing domain of fibronectin binds to the IIIa subunit. All these cell surface complexes have the same general rather unusual structure (see figure). On reducing SDS gels the two (or more) subunits run as one band or as a doublet at a relative molecular mass of about 140,000. On non-reducing gels they appear as well-separated bands. This is because the lower band runs more slowly under reducing conditions (typical of proteins containing intramolecular disulphide bonds), while the upper bands (which I shall call IIb-like) run faster under reducing conditions. For the platelet IIb band this behaviour is caused by the release of a small com-

ponent on reduction⁸; it is likely that all IIb-like components will have such a light chain attached.

What do all these receptors have to do with each other? Their fibronectin-binding activity (shown directly for some and inferred from the adhesion-blocking activities of the antibodies for the others) and their common structure suggest that they are all the same type of receptor. However, the differences between them indicate that there does not seem to be such a thing as *the* fibronectin receptor, but rather a whole family of related but subtly different receptors. They differ in molecular mass, glycosylation and num-



Migration of the platelet fibronectin receptor IIb/IIIa on reducing (R) and non-reducing (NR) polyacrylamide gels. LC, light chain. (After ref. 8.)

ber of components. Until direct comparisons of the proteins are made it is futile to speculate whether the differences in mass simply result from variations in gel conditions, but some are so great that they probably reflect real differences between receptors from different cells.

A family of cell surface glycoproteins in which such cell-specific differences do occur are the PS antigens in the fruitfly *Drosophila*, which have the same unusual structure as the fibronectin receptors⁹. Found in most tissues of the body, these complexes each consist of one IIa-like component and one or more IIb-like components. The IIIa-like component is present in every complex whereas the IIb-like components attached to it vary between tissues⁹. PS antigens are thought to be involved in the segregation of cell populations during development, so it will be interesting to see if they are functionally similar to vertebrate fibronectin receptors.

Most importantly, fibronectin receptors differ in their affinities and fine specificities. In fact, these receptors are only a subset of a larger family of receptors for proteins containing the RGD recognition sequence (for example, vitronectin,

laminin and fibrinogen). Cells can carry two separate receptors with different specificities, or they can use one receptor to bind to more than one ligand. The first case is exemplified by osteosarcoma cells¹⁰, which have two different receptors for fibronectin and vitronectin, neither of which binds laminin. The binding of both receptors to their ligands is inhibited by peptides containing the RGD sequence. On the other hand, the receptor on fibroblasts can bind to both fibronectin and laminin³. Platelets appear to use a single receptor to bind fibronectin and fibrinogen, because fibrinogen inhibits binding of fibronectin to platelets. Finally, receptors for the same ligand on different cells can differ in their affinities. In contrast to the molecules isolated by affinity purification, the receptor isolated by one of the antibodies is not retained on fibronectin columns, but only retarded (compared with its elution from bovine serum albumin columns)⁶. Such weak binding is not unexpected, as receptors which cells use for migration should bind and be readily detachable from the substrate.

The RGD receptors may use the same trick that is developed to perfection by the immune system and that might be used by olfactory neurones¹¹, namely to use receptors with constant structure but variable specificities to handle diverse ligands in the environment. If all these receptors bind to the RGD sequence, how is their fine specificity determined? They all consist of more than one component, only one of which seems to bind to the RGD-containing domain, if the finding for the platelet IIIa band can be generalized. Perhaps this subunit mediates the binding to the recognition sequence RGD, while the other associated components determine the fine specificity of the receptor. These components might affect the conformation of IIIa and thereby modulate the binding site, so that RGD is recognized only in some contexts but not in others; they might combine to form different binding sites; or they might themselves carry a second binding site specific for another epitope on the target molecule. Such variation of receptor specificity could provide a guiding and sorting mechanism for cells during morphogenesis. □

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Stratospheric chemistry

Depletion of Antarctic ozone

from A. F. Tuck

THE total amount of ozone overhead in late winter and early spring at Antarctica has decreased by about 40 per cent during the past decade¹. Recent analysis of satellite data² shows that the phenomenon affects much of the cold, cyclonic vortex found there, and that column abundances are among the lowest recorded anywhere on the globe. A time series of ozonesonde profiles³ taken in 1982, when compared with data taken in the late 1960s and early 1970s, suggests that much of the decrease occurs in the lower stratosphere, at and below the altitudes where maximum number densities of ozone occur (Fig. 1). These experimental findings are a major surprise, and are taxing the theoretical ingenuity of stratospheric researchers. The first calculations of these late winter and spring antarctic ozone reductions appear on pages 755 and 759 of this issue^{4,5}. Until an experimentally validated mechanism for these reductions is available, the implications for the global ozone layer are not clear.

The work reported in refs 2, 4 and 5 was presented for the first time at a recent meeting*. Some simple numerical model calculations demonstrate the extent of the ozone deficit caused in southern mid-latitudes as poleward motion of ozone-rich air over Antarctica, largely suppressed during winter, occurs in spring and fills in the 'ozone hole' in the cold vortical air by mixing with it (R. Rood, NASA Goddard Space Flight Center). This induced mid-latitude deficit is small; the model calculation is based on simplified large-scale dynamics, with no photochemistry or radiative transfer. The antarctic winter polar vortex in the stratosphere is approaching the radiative equilibrium temperatures computed for air with 'climatological' O₃, CO₂ and H₂O as the gaseous absorbers and emitters; general circulation models indicate that under these circumstances the vortex can be quite sensitive to comparatively weak perturbations (J. Mahlman, NOAA Princeton Geophysical Fluid Dynamics Laboratory). Finally, I reported UK Meteorological Office calculations of radiatively corrected isentropic trajectories over both arctic and antarctic winter polar caps in 1979, which show that over a 10-day period, air parcels starting poleward of 75° latitude in the Arctic tend to encounter sunlight within a few days. In Antarctica, however, most air parcels stay in the dark

for the entire 10 days (Fig. 2).

At the meeting, results were described of forward and back trajectories initiated by observations of arctic polar stratospheric clouds by the SAM II instrument⁶. Coincident observations of O₃, NO₂, HNO₃ and H₂O by the LIMS instrument⁷ on Nimbus 7 in January 1979 are also available. Preliminary results show ozone

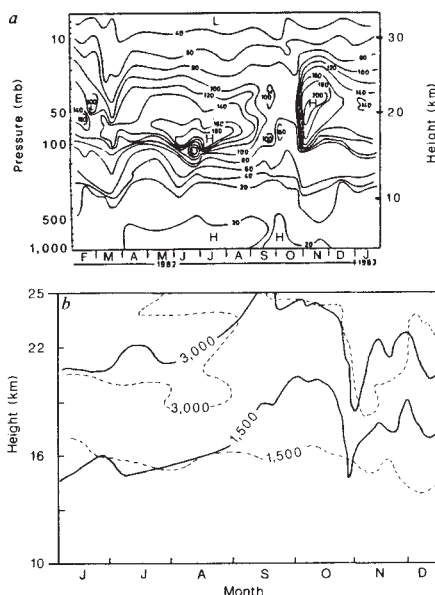


Fig. 1 *a*, Partial pressure of ozone over Syowa (69°S, 40°E) during 1982, from 22 ozonesonde ascents. Contours are in nanobars. (Redrawn from ref. 3.) *b*, Contours of ozone volume mixing ratio (p.p.b.v.) over Syowa (69°N, 40°E) during the last half of 1970 (broken lines) and 1982 (continuous lines). Note the coherent behaviour of the 1,500 and 3,000 contours in 1982 over the whole period, in contrast to the behaviour in 1970. Redrawn from plots supplied by S. Solomon (personal communication).

and nitric acid reductions along the forward trajectories which are fast enough to be consistent with the Antarctic data; the scatter means that the results are barely at the 2σ level. The results depend critically on removing the effects of cloud from the LIMS data.

There was discussion of the general requirements for a ground-based network for early detection of stratospheric change, particularly in relation to Antarctica. Several techniques for measuring the contrasting conditions in the upper mid-latitude stratosphere and the lower polar stratosphere were discussed, prominent among them ground-based laser radars (lidars) and passive microwave millimetre and sub-millimetre radiometers. One instrument, a high-resolution infrared interferometer that scans the spec-

trum from 2- to 16-μm wavelength using absorption of solar radiation, offers the possibility of detecting and measuring the column abundances of many different stratospheric molecules.

The planned campaign for austral spring 1986 includes flights of ozone balloon-sondes from the US antarctic stations at McMurdo and Amundsen–Scott. There will be a supply flight (sponsored by the National Science Foundation) into McMurdo on 21 August, and it is hoped to fly in scientists to measure ozone and aerosols (D. Hoffman, University of Wyoming); ClO (P. Solomon and R. de Zafra, State University of New York at Stony Brook); NO₂ and OCIO (A. Schmeltekopf, G. Mount and S. Solomon, NOAA Aeronomy, Boulder); and instruments such as a high-resolution absorption FTIR spectrometer (C.B. Farmer, NASA Jet Propulsion Laboratory). There will also be measurements of NO₂ column amounts at three New Zealand stations, and British Antarctic Survey ozone measurements will continue. A more extensive Antarctic campaign, which may include aircraft and balloon experiments in addition to the ground-based instruments, is planned for austral spring 1987. There is clearly a need for a small network aimed at early detection of trends in the key molecules, but there is at present no obvious source of funds for the long-term commitment required.

Several points emerged from the meeting. First, and possibly the most important, is that it is difficult to find any reliable data in the chemical kinetics literature on gas–ice crystal interactions in the atmospheric conditions of the lower antarctic winter stratosphere. It is also true that the relevant homogeneous gas-phase rate coefficients involve a considerable extrapolation to reach the temperature range 165–225 K. We simply cannot be sure of the chemistry in this regime, particularly concerning the larger reservoir species like N₂O₅ and ClONO₂, and molecules such as BrO. Therefore, laboratory measurements are as essential as those in the atmosphere.

Second, it is not clear what substituting ice crystals for water vapour (which is what happens as temperature falls below about 192 K) does to the radiative balance, although there has been a calculation of this effect⁸.

Third, it seems probable that a reliable calculation of the dynamical-radiative balance is essential, presumably by a yet-to-be developed general circulation model which handles the stratospheric polar night vortex and its low temperature adequately. The coherent behaviour of the 1,500 and 3,000 parts per 10⁹ by volume (p.p.b.v.) contours over the entire second half of 1982 at Syowa (Fig. 1b), a pattern not evident in 1970 or earlier, suggests that attention should not be confined to

*Antarctic Ozone meeting, Boulder, Colorado 4–7 March 1986. Sponsored by National Aeronautics and Space Administration (NASA), National Oceanographic and Atmospheric Administration (NOAA) and the Chemical Manufacturer's Association.

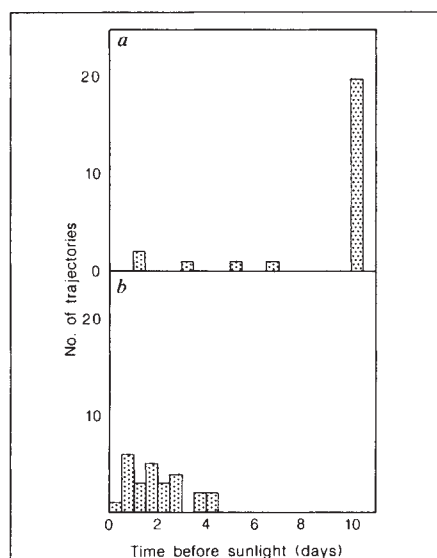


Fig. 2 Numbers of 10-day trajectories starting in the polar caps (75° – 90° latitude) and staying in the darkness for differing lengths of time. Twenty-five parcels evenly distributed over the cap were considered, using the quasi-isentropic approach of Austin and Tuck⁹. *a*, Antarctica 18–28 June 1979; *b*, Arctic 12–22 January 1979. Eighty per cent of the antarctic parcels had not experienced sunlight at the end of 10 days, whereas all the arctic ones had done so after 4–5 days.

September and October. It may also mean that the polarization of theories so far, into dynamical and chemical categories, may be premature — it seems likely that both sorts of process are involved. If chemistry on ice-particle surfaces at very low temperatures (-80°C or colder) is important, then it may be worth looking more closely at those locations in the tropical lower stratosphere where large cumulonimbus storms are believed to maintain extensive anvil cirrus sheets at the low temperatures, by moist convection rather than by radiative cooling. The different exposure to solar radiation at the two places may yield important clues.

Finally, it should be apparent from the above that model calculations of this effect are as yet in their infancy, and are no better than the input physico-chemical data. There is a long way to go experimentally before an accepted qualitative, let alone quantitative, explanation is available. □

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Molecular biology

Globin gene monkey business

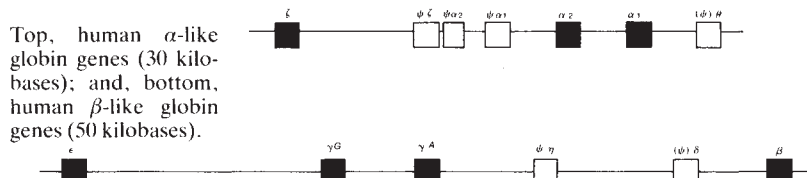
from Nick Proudfoot

THE discovery of a new α -like globin gene in orang utan, reported by Marks, Shaw and Shen on page 789 of this issue¹, and the possibility that a similar gene has until now escaped notice in humans, is an illustration of how there are still many surprises in store even in a well-characterized gene system such as globin. It is, however, possible that the orang utan θ -globin gene is inactive — in other words, a pseudogene — because it may not possess a functional promoter sequence. Although θ -globin could have little significance to the orang utan, it is possible that in other primates, man included, the new globin gene has an important but as yet undetected biological role. The characterization of θ -globin in other primates is therefore eagerly awaited to see whether a new functional θ -globin gene will be identified.

Globin genes were among the first to be mapped and sequenced in eukaryotes². Tidy clusters of either α - or β -like globin genes (see figure) are now familiar in mol-

sequenced intergenic regions between ζ and $\alpha 2$ (ref. 8).

To this collection we must add the θ -globin gene at the downstream end of the α -gene cluster that is described in this issue¹. In the orang utan this gene has the usual arrangement of three exons that would encode a fairly standard α -like globin polypeptide chain. Furthermore, most of the amino acids common to the α -globins of different mammals are found in θ -globin, and those that are not found fit with a β -like globin sequence. Even so, it seems unlikely that this gene can be active because its promoter sequence elements, the CCAAT and ATA boxes, are moved more than 100 nucleotides away from their normal position by the insertion of a GC-rich sequence. Such alterations to the spacing of the θ -globin promoter would be expected to prevent initiation of transcription. Although the θ -globin gene is probably non-functional in orang utan, it may still be active in humans. Indeed, there are possibly a few



ecular biology textbooks. The human globin gene clusters are especially well characterized as globins at each developmental stage have each been assigned their gene and many mutations from different thalassaemias have been pinned down to specific positions on the globin gene map³. In addition to functional genes, there are a surprisingly high number of apparently functionless pseudoglobins, particularly in the human α -globin gene cluster. At the last count (see figure), there are three pseudogenes between the embryonic ζ -globin and the two adult α -globin genes. These pseudogenes cover the whole spectrum of gene inactivity. The $\psi\zeta$ gene looks quite healthy and, were it not for a nonsense mutation at codon 6, might be expected to produce ζ -globin⁴. In some human populations, $\psi\zeta$ -globin has a normal codon 6 but surprisingly still fails to produce a gene product⁵. The gene $\psi\alpha 1$ is very clearly non-functional, as a whole set of mutations prevent both its translation and its transcription^{6,7}. The newly identified pseudogene, $\psi\alpha 2$, is so extensively diverged from α that it was only picked up by a computer search for α -like sequences in the recently completely

as yet unassigned embryonic globins, as shown by the recent analysis of very early human embryos⁸. What the new work of Marks *et al.*¹ does show is that θ -globin in orang utan is quite similar to a rabbit α -globin pseudogene, suggesting a very early evolutionary origin for θ -globin.

A similar situation to θ -globin exists in the human β -like globin gene cluster. Here, only one pseudogene, the $\psi\beta 1$ gene, has been unequivocally identified¹⁰, although the low level of δ -globin gene activity could be taken as an argument that δ is also effectively a $\psi\beta$ gene¹¹. Interestingly, $\psi\beta 1$ has strongly con-

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served counterparts in other primates and even in more distant evolutionary relatives such as goat, where it is a functional embryonic globin gene. Indeed Harris *et al.*¹² renamed $\psi\beta$ η -globin to emphasize that it does have a function in some mammalian globin gene systems but that this function has been lost in primates. It may be similar with θ -globin, where the gene is probably functional in some mammals but possibly is not in primates.

What these studies on globin genes and pseudogene in mammals clearly illustrate is that globin gene evolution has produced a very flexible set of genes capable of providing the individual needs of each species for oxygen carriage at each developmental stage. Every newly created globin gene is

then randomly mutated and the mutations either selected for functional globin variants or are put on the gene scrap-heap as useless variants. Studies of θ - and η -globin further show that previously useful variants may subsequently cease to be so and thus will end up with other non-functional duplicated globin genes in the scrap-heap. The title of a paper describing the evolutionary path of the δ -globin gene as "the rise and fall of a globin gene"¹¹ is equally applicable to θ - and η -globin genes and it might turn out to be applicable to other gene families. □

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Neurobiology

Clues about glues in development

from Colin J. Barnstable

FORMATION of a complex nervous system from a simple neuroepithelium involves many events of regionalization, cell migration, cell death, directional axon growth and specific cell interactions. It has often been proposed that molecules at the cell surface mediate the interactions between the genetic and the epigenetic processes of neural development. It has been thought such molecules can be recognized by their ability to cause adhesion between cells. Several recent papers have suggested ways in which known cell surface molecules might interact with each other and with extracellular matrix molecules to control important events, such as synapse formation, in neural development.

Probably the best-characterized cell adhesion molecule is N-CAM^{1,2}. This transmembrane glycoprotein appears after neural induction in the embryo. It is expressed by all neurones, some glia and a few other cell types in the adult and causes cell adhesion by homophilic interactions between domains in the amino-terminal portion of the molecule. It has become clear over the past few years that many other molecules detectable by adhesion assays appear during development of the nervous system but the mechanism of action of many of them remain obscure.

The potential functions of cell adhesion molecules in the outgrowth of axons has been studied intensively. There is now evidence that some aspects of directed axon growth might be controlled through N-CAM. The simplest model for this would be one in which a track of an adhesive molecule such as N-CAM is laid down in the tissue. An immunocytochemical study of the developing chick optic tract suggested that the retinal ganglion cell axons were following a track of N-CAM expressed on the endfeet of

radial glia³. Although an attractive model, its general applicability to pathway formation is not clear. Other factors are almost certainly operating, as retinal ganglion cells placed in ectopic sites can send axons along very abnormal pathways yet still reach their correct targets⁴.

A second class of model has arisen from studies on the innervation and reinnervation of muscle. N-CAM is present on the surface of myotubes in the embryo, but is lost as development proceeds and is almost absent from adult muscle. Denervation or paralysis of adult muscle leads to an increase in N-CAM levels over the whole myotube until synapses are re-established. N-CAM is also associated with interstitial cells between the muscle fibres⁵. But the increase in N-CAM expression over the whole muscle cell does not explain why synapse formation during reinnervation preferentially takes place at the old synaptic sites. In studying the

changes in other molecules after denervation, Sanes and co-workers found that fibronectin, a heparan sulphate proteoglycan and an extracellular glycoprotein J1 also accumulated in the interstitial spaces near the denervated synaptic sites⁶.

These molecules, particularly J1, have all been shown to mediate adhesion of neurones to other cells or to basement membranes. The findings led the authors to suggest these molecules may act with N-CAM to provide a hierarchy of cues that guide axons towards the old synaptic sites (see Fig. 1). McMahan and colleagues have described a molecule in the extracellular matrix that may mark the old synaptic sites and perhaps this type of molecule represents the final step in the hierarchy⁷. Thus, the control of axon growth may involve a generalized interaction mediated by N-CAM and then more specialized interactions mediated by N-CAM plus other molecules. Whether this hierarchy represents quantitative levels of adhesivity or qualitative differences in the signals provided by the different molecules is not yet clear.

Recent reports by Glaser and co-workers suggest that some aspects of such an adhesive hierarchy may be caused by modulations of N-CAM itself. Adhesion

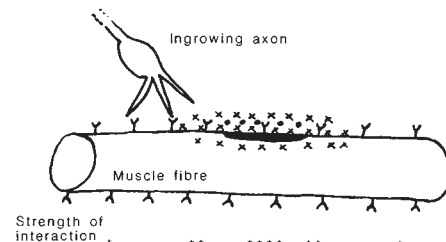


Fig. 1 The growth cone of the ingrowing axon can detect the molecules on the muscle surface and in the interstitial space. Its growth is directed up the gradient of increasing strength of interaction that leads to the old synaptic site. Y, N-CAM; x, J1, fibronectin and heparan sulphate; ●, synapse-specific molecules; ■, old synaptic site.

100 years ago The Hong Kong meteorological observatory

This first-class meteorological observatory was erected in 1883, and the regular work of observing began on January 1, 1884. Weather Reports appear monthly, and we have now before us the observations and work of Mr Doberck and his staff for the first two years. For the first two months the work was restricted to eye-observations, but meanwhile no time was lost in erecting the barograph, thermograph, anemograph, pluviograph, and sunshine recorder, which are similar to those in use at Kew; and from April 1, 1884, the Monthly Reports include a continuous hourly record of the more important elements of the climate of Hong Kong. The buildings are erected on the peninsula of Kaulung, facing the harbour, on the top of Mount Elgin, a small eminence rising from the plain to a height of about 100 feet

above mean sea-level. It may also be noted that the ground has been carefully turfed where the instruments are placed. In addition to the usual tabulations and their averages, the Monthly Report gives a carefully observed log of non-instrumental phenomena, such as dew, fog, unusual visibility, halos, and thunderstorms.

The results show that the amplitude of the daily range of the barometer is greatest from November to February, when the rainfall is least and their air driest, the mean difference during these four months between the morning maximum and afternoon minimum amounting to 0.102 inch. On the other hand, the mean of the four months from June to September when the monthly rainfall nearly equals 12 inches, only amounts to 0.069 inch. The diurnal range of temperature is small, being for the year only 5°·5, the maximum, 72°, occurring in December, and the minimum, 4°·0, in February.

From *Nature* 33, 148; 17 June 1886.

of chick neural retina cells to extracellular matrix material was inhibited by an antibody against N-CAM and by heparan sulphate⁸. It has now been shown that N-CAM itself has a binding site for a heparin-like proteoglycan⁹. Thus binding of neurones, and perhaps neuronal processes, to extracellular matrix may result from a N-CAM–proteoglycan interaction.

As heparan sulphate has been shown to be on the cell surface of retinal cells, Glaser and co-workers asked whether it might also have a role in the N-CAM-mediated cell–cell adhesion. They found that binding of chick neural retina cells to retinal monolayers was inhibited by heparin but not an unrelated proteoglycan¹⁰. This cell–cell adhesion was also inhibited by the isolated heparin-binding domain of N-CAM and by an antibody that recognizes this domain. As binding of heparin alters the susceptibility of N-CAM to proteolytic cleavage it is thought that the interaction involves a

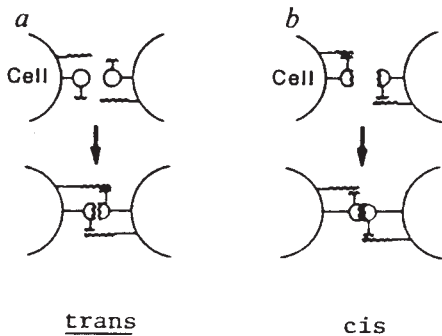


Fig. 2 Possible modes of interaction between heparan sulphate (—) and N-CAM (—○—). *a*, In a *trans* interaction the heparan sulphate on one cell can interact with the N-CAM on another cell. *b*, in a *cis* interaction the heparan sulphate binds to and possibly modifies the N-CAM on the same cell. (From ref. 10.)

conformational change in N-CAM. What is not yet clear is whether the proteoglycan on one cell interacts with the N-CAM on another cell, or whether the proteoglycan interacts with N-CAM on the same cell and thus affects the affinity of the N-CAM–N-CAM homophilic interaction between cells (see Fig. 2).

These results lead to several conclusions and speculations. One is that the distinction between cell–cell adhesion and cell–substrate adhesion is becoming less distinct. Molecules such as N-CAM may function in both. A second is that

events such as cell adhesion or synapse formation may require several different molecules, and the affinity of each interaction might be modulated by various cellular and extracellular components. Finally, as interactions between adhesion molecules can lead to conformational changes in transmembrane glycoproteins, these molecules may do much more than glue cells together. Cell adhesion, as measured by binding assays *in vitro*, may be an experimental epiphenomenon

rather than a primary driving force in development. Cell interactions through different adhesion molecules may be another source of information — with growth factors and environmental signals — that a cell receives, integrates and translates into the appropriate survival, movement and differentiation response. □

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Ichnology

Sedimentological use of dinosaurs

from Michael J. Benton

In films and in fictional literature, massive brontosauri are pictured crashing through the undergrowth, felling trees as they go, creating seismic tremors as each heavy foot meets the ground. New studies of fossilized dinosaur footprints show how they can be used by geologists to determine ancient water depths, current flows and the slopes of sand dunes. A review¹ of these studies launches the journal *Palaiois*, which will be devoted to research on sedimentological and palaeoecological aspects of the fossil record.

Dinosaur footprints are known from dozens of localities around the world. Most research effort so far has been devoted to determining the taxonomy of the tracks and to identification of the track-makers. Specimens produced by every kind of dinosaur have been found, ranging from the tiny bird-like footprints made by small theropods to the vast stump-like marks made by sauropods such as *Brontosaurus*. Palaeobiological interpretations of these trackways have also been made. In many places, for example, multiple tracks show that herds of dinosaurs walked in close formation in the same direction^{1,2}. It has also been possible to calculate how fast the dinosaurs were walking when they made the tracks. The size and spacing of individual prints within a trackway give estimates of speed according to a simple formula³. The fastest 'ostrich' dinosaurs could run as fast as 35–60 km h⁻¹, whereas *Brontosaurus* would have blundered along at 12–17 km h⁻¹ (ref. 4).

The new work on dinosaur footprints focuses on their use in sedimentology. Several authors, for example, have suggested that dinosaur tracks are found largely along the edges of water bodies in close proximity to the strand line^{1,5,6}. The tracks can even be used to reconstruct the exact position of a marine or lacustrine shoreline by measuring the directions of locomotion, which are assumed to be roughly parallel to the shore. In some cases, the footprints within a single trackway vary in depth, the depth of imprint

varying with the water content of the sediment — shallow prints are assumed to indicate dry land and deep prints to indicate where the dinosaur was paddling through shallow water.

Dinosaur tracks can also be used to estimate water depth accurately. If the animal was paddling, the water cannot have been deeper than head height — about 2–3.5 m for a *Brontosaurus* with its neck held horizontally. In other cases, a normal trackway breaks down in deeper water as the animal starts to swim, just touching down on the bottom with a stray foot for steering. There are several examples of sediments which might have been interpreted as having been deposited in deep water but for the dinosaur footprints¹. The direction and power of water currents can also be estimated by observing how a trackway veers sideways from the animal's intended direction of travel (shown by where its toes point). Dinosaur footprints formed on dry land give an indication of the palaeoslope, the slope of the land at that time⁷. On walking up a sand dune, heaps of sand are pushed back by the heels, and on walking downhill, the toes dig in deeper than normal.

What of the smashing and crushing of the habitat caused by dinosaur locomotion? In one case, a brontosaurus trampled across a collection of living unionid clams in shallow water and preserved them for posterity in the base of the huge footprints¹, a sedimentological phenomenon termed *dinoturbation*⁸. □

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Portuguese role in spread of HTLV-I virus

SIR—Having arrived in Tokyo recently, I thought that I had learnt another word to improve my Japanese vocabulary, by reading Gallo *et al.*¹ on the origins of human T-lymphotropic viruses. However, the word *amakawa*, which they inform us is the Japanese word for monkey, is absent in recent reference sources² I have checked. Even my Japanese colleagues in the laboratory seem unaware of it as an alternative term for monkey. The popular word for the Japanese macaque monkey is *saru*. The monkey deity in Japanese mythology is known as *sarutahiko*, while a monkey show, once a common street entertainment, is termed *sarumawashi*³.

Rosenior's comment, "the presence of the HTLV-I virus in the vast expanse of continental Asia cannot be ruled out due to lack of epidemiological studies in the area"⁴, seems to have an element of truth, if we believe the historical records of the seafaring adventures of the Portuguese. Figure 1 of Wong-Staal and Gallo⁵ neglects (or slightly distorts) the travel routes taken by the Portuguese in the early part of the sixteenth century. Vasco da Gama landed in Calicut, South India in 1498⁶ and, in 1505, a Portuguese fleet commanded by Lourenco de Almeida was blown into Colombo, Ceylon, by adverse winds⁷. Malacca fell to the Portuguese in 1511. It was only after establishing contacts in India, Ceylon and Malacca that the Portuguese arrived in Japan, in 1543.

Prior to his arrival in Japan in 1549, Saint Francis Xavier first disembarked in Goa, India, the centre of Portuguese activity in the East in 1542 and spent the next three years on the southeastern coast of India among the Tamil-speaking pearl fishermen, the Paravas⁸.

According to Percival Spear⁹, in those times the Portuguese relied greatly on sea power based on fortified posts and backed by settlements; however, Portugal, with less than one million people, was desperately short of manpower. Therefore, intermarriage was encouraged between the Portuguese seafaring adventurers and the natives of the Indian subcontinent. That practice was the origin of the mixed population of Luso-Indians (or Goanese) along the western coast of India and in Ceylon.

If it is believed (as mentioned by Gallo *et al.*¹) that the Portuguese came to Japan with monkeys, it is highly probable that the monkeys would have been from the Malayan region and not from Africa. (South-East Asia is also the endemic zone of apes such as gibbon, siamang and orang utan.) Historical records reveal that Portuguese trade was directed mainly

between Japan and China rather than Africa.

Therefore, it is my hypothesis that the dark-skinned people who were taken to Japan by Portuguese were the South Indian Tamils of fishermen caste, and not Africans, as suggested by Gallo *et al.* The linguistic relationship between the Tamil language and Japanese language has been studied by Ohno⁹.

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Commercial samples of subtilisin BPN'

SIR—The serine protease subtilisin BPN' (also known as novo or nagarse) from *Bacillus amyloliquefaciens* has been cloned and expressed on three separate occasions¹⁻³. We have found a discrepancy of over a factor of 10 between the activity of the cloned enzyme and the activity of commercial enzyme purchased from Sigma Chemical Company (Protease Type VII, P 5255, from *Bacillus amyloliquefaciens*). The cloned enzyme has identical activity to an authentic sample of nagarse enzyme which was used for the original crystallographic studies⁴. (This sample was given to A.R.F. by Dr C.S. Wright in 1969.) Kinetic and physical properties indicate that the commercial enzyme is probably subtilisin Carlsberg, isolated from *Bacillus licheniformis*. We reported our findings to Sigma who have now conducted their own tests. They have just informed us that they agree with our findings and that, for the past ten years, their subtilisin BPN' has not been the authentic enzyme.

The hydrolysis of the synthetic substrate succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl *p*-nitroanilide by the cloned enzyme is characterized by values for k_{cat} and K_m of 57 s⁻¹ and 0.15 mM respectively (25 °C in 0.1 M Tris-HCl buffer, pH 8.6), the enzyme concentration being measured by active-site titration. These are identical to the values for the independently cloned gene¹. Under the same conditions, the values for the authentic nagarse enzyme are essentially identical at 60 s⁻¹ and 0.15 mM. The values of k_{cat} and

K_m for the Sigma subtilisin BPN' are 642 s⁻¹ and 0.255 mM respectively. This enzyme is obviously not subtilisin BPN'. The commercial subtilisin Carlsberg from Sigma (Protease Type VIII, P 5380) has similar properties to the commercial subtilisin BPN': k_{cat} is 938 s⁻¹ and K_m is 0.234 mM. We also find that the pK_a of the active site of the commercial sample of BPN' is identical to that of the Carlsberg enzyme and different from the value found for both the authentic and cloned samples³. Sigma have now performed amino-acid analyses and isoelectric point determinations and find that they are inconsistent with their enzyme being subtilisin BPN'.

It has just been reported⁵ that subtilisin BPN' obtained through Serva has the same sequence and crystal structure as subtilisin Carlsberg (the sequence of genuine BPN' being about 70% homologous to Carlsberg). It is likely that further preparations of subtilisin BPN' marketed by other suppliers are similarly dubious, not least because subtilisin BPN' from Sigma is prepared for Sigma and not by them. Subtilisin BPN' is also supplied to the food industry and so such users may now wish to check that they are using the correct enzyme.

Much of the work which has been performed on commercial subtilisin BPN' over the past ten years must be re-evaluated in light of the above, especially those studies comparing commercial subtilisins BPN' and Carlsberg⁶.

We thank Sigma for their concern and help in resolving the problem.

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Alu sequences in the LDL receptor messenger RNA

SIR—*Alu*-like sequences, which are highly conserved in eukaryotes, are found, for instance, in the signal recognition particle^{1,2} where, in conjunction with a 9/14K protein they arrest the synthesis of pre-secretory proteins at the level of elongation according to the recent work of Siegel and Walter³. At the end of their paper, Siegel and Walter made an interesting suggestion about the possible function of the *Alu* sequences in the mRNA of the

low density lipoprotein (LDL) receptor in its translational control. We wonder if this control is of major importance.

Sequence homology between human and bovine LDL-receptor mRNAs is substantial; however, unlike the human mRNA¹, the bovine mRNA does not contain *Alu* repeats⁴. Indeed, Hobbs *et al.*⁴ reported that the genetic events that gave rise to the *Alu* cluster occurred within the past 33 million years, before the development of the Hominidae. Animal species which diverged earlier from human ancestors (for example, cow, rabbit or baboon) lack *Alu* sequences in their LDL mRNA. Despite this, there is no evidence that regulation of the LDL receptor in these species differs substantially from that in the human⁵⁻⁸. Accordingly, it becomes unlikely that *Alu* sequences play a major regulatory role in the translational control of the LDL receptor. Definite proof of a regulatory role of the *Alu* sequences in LDL mRNA awaits studies comparing the regulation of the LDL receptor produced in the presence or the absence of *Alu* sequences in the 3' untranslated region of LDL mRNA.

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How to test special relativity

SIR—Psimopoulos and Theocharis¹ suggest that the Michelson-Morley experiment should be repeated in space to ensure that the light reference frame is not, in fact, locally determined by electromagnetic or gravitational field effects attributable to the physical size of the Earth. Relativity recognizes only the role of the observer as the determining factor for the applicable frame of reference.

There is certainly merit in this proposal, especially as rotation can be detected by Michelson interferometer techniques (the

ring laser gyro) and such rotation sensors will undoubtedly find practical application in space vehicles. It is logical to verify whether the Michelson interferometer can sense translational motion of a space vehicle, once it has moved outside the Earth's influence.

However, the underlying hypothesis of Psimopoulos and Theocharis leaves one wondering how small the ambient field effects have to be in order not to control the electromagnetic reference frame. In this regard it should be remembered that the Michelson-Morley experiment of 1887 antedates Wiener's 1890 discovery of the existence of stationary light waves when a ray of light is reflected back upon itself by a mirror. This means that in the Michelson experiments the incident ray is passing through the field of the reflected ray. We assume that this field does not affect the speed of the incident ray. If this assumption is wrong and field energy can present, in effect, a refractive index modifying the speed of light, then without appealing to the ambient effects of an Earth field we can even consider that the optimization of the energy in the standing wave system will determine the local frame of reference for light speed. Indeed, unless the action is to cause the incident light speed relative to the mirror to be identical to that of the reflected light speed, there will be an amplitude modulation of the standing wave pattern and the energy will be deployed unevenly along the ray path. It seems possible that the standing wave energy would adopt the motion of the system and assure uniformity, thus making the light-speed isotropy a forced condition of the apparatus.

Thus while Psimopoulos and Theocharis may well be correct in regarding the strong influence of the Earth as the dominating consideration, there is a possibility that a weak ambient field is sufficient. In this case the Michelson-Morley experiment cannot give any valid indication concerning motion through Maxwell's ether and this will hold also for free flight in space. Note also that a wave is not reflected back on itself in the ring laser gyro. Here, any standing waves are set up in a non-rotating frame and are not locked into the rotary motion. Clearly, therefore, the objectives of the Michelson experiments have not been met for translational motion until one devises an optical configuration that works without having light rays reflected back along the same path. Such experiments have not yet been performed in the laboratory, but are, in principle, feasible².

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PSIMOPOULOS AND THEOCHARIS REPLY — Aspden gives a valid theoretical objection to our proposal that the Michelson interferometer might detect its translational movement through the assumed electromagnetic ether. This seems to be a sound, though not conclusive, criticism. It is true that, unlike in the Michelson interferometer, in the ring laser gyro a ray is not reflected back on itself by a single mirror. Nevertheless, a ray is indeed reflected back on itself by a combination of optical devices. Thus everywhere in the ring laser gyro, just like in the Michelson interferometer, every ray is passing through the field of a ray propagated in the opposite direction. Therefore standing waves are set up in the ring laser gyro too (in the non-rotating frame), but they have not been observed to be locked into the rotary motion (except perhaps for very slow rotations). Likewise, standing waves are indeed set up in the Michelson interferometer (in the non-moving frame), and we would expect that they will not be locked into the translational motion. Of course the ultimate judge is the experimental test, which however has never been performed.

The practical application of optical rotation sensors having been mentioned, we note that such a sensor is in an advanced stage of development: Standard Telecommunication Laboratories have recently announced (*Electronics Power* **32**, 111; 1986) that they expect to have a marketable fibre-optic gyro available within two years. The practical issue which we would like to raise is: Why is there no laboratory in the world working on the development of optical translation sensors? The answer is the almost universal acceptance of the special relativistic postulates, and the consequent lack of interest in directly testing them experimentally. It is quite possible that this lack of interest might be preventing not only theoretical advancements, but also, as presently indicated, important technological applications. This is the chief reason why we insist that the fundamental assumptions themselves, rather than their mere inferences, should be directly and constantly tested.

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a rather technical character which are not provoked by articles or by letters previously published (where the Matters Arising section remains appropriate).

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Biologists under control

Sheldon Krimsky

The Politics of Uncertainty: Regulating Recombinant DNA Research in Britain.

By David Bennett, Peter Glasner and David Travis.

Routledge & Kegan Paul: 1986. Pp.218. £20, \$35.

Cloning and the Constitution: An Inquiry into Governmental Policymaking and Genetic Experimentation.

By Ira H. Carmen.

University of Wisconsin Press: 1986. Pp.223. \$22.50, £27.

In February 1986, eleven years to the month after the international meeting on the risks of recombinant DNA (rDNA) research was convened at Asilomar in Monterey, California, citizens of that county stopped the first federally authorized field test of a genetically modified bacterium. The California biotechnology company Advanced Genetic Sciences had received approval from the US Environmental Protection Agency and the National Institutes of Health (NIH) to spray the bacterium on a strawberry patch as a means of assessing its potential for inhibiting ice-nucleation. Local residents successfully opposed the release on the grounds that the risks were unknown and that no effort had been made to inform the community.

What a strange coincidence! "Asilomar 1975" was the starting point of the first generation of debates on the safety and regulation of rDNA technology. Now, as the technology reaches the stage of agricultural use, a new public controversy has emerged at the same place. This time the issue is over the intentional release of genetically engineered products into the biosphere.

Science and politics are still playing out the legacy of "Asilomar 1975" as gene splicing takes new turns in its journey towards public acceptance. No event since the release of thermonuclear energy, in the form of the atomic bomb, has fostered such intense discussion about the responsibility and accountability of science to society. The questions are twofold. If faced with uncertainties about the safety of their research, will scientists ever again expose their collective anxieties to the public; that is, has Asilomar and its aftermath driven scientists into a shell? And what has the experience of the rDNA controversy taught us about science and democracy?

The two books reviewed here provide a fresh set of perspectives on these matters. *The Politics of Uncertainty* is the first book-length investigation of British policy on rDNA research; cast in a sociological framework, it is rich in historical detail and filled with insightful comparisons of British and American policy-making. *Cloning and the Constitution* examines selected periods of the rDNA debate in

the United States through the prism of the author's unique construction of US Constitutional theory. While Bennett, Glasner and Travis are concerned about the public's role in the formulation of science policy, Carmen emphasizes instead the rights of scientists to engage in research. This dichotomy expresses the essential tension underlying the DNA

RECENT advances in techniques for the isolation and rejoining of segments of DNA now permit construction of biologically active recombinant DNA molecules in vitro. ...

There is serious concern that some of these artificial recombinant DNA molecules could prove biologically hazardous. One potential hazard in current experiments derives from the need to use a bacterium like E. coli to clone the recombinant DNA molecules and to amplify their number. E. coli strains commonly reside in the human intestinal tract, and they are capable of exchanging genetic information with other types of bacteria, some of which are pathogenic to man. Thus, new DNA elements introduced into E. coli might possibly become widely disseminated ... with unpredictable effects. ...

Warning shot — extract from the letter from Berg *et al.*, July 1974, recommending the deferential of various types of rDNA experiment.

controversy; namely, the struggle between the self-governance and the social control of science.

In gathering material for *The Politics of Uncertainty*, the authors were at a considerable disadvantage compared to their counterparts who have studied the corresponding events in the United States. British policy-making essentially took place behind closed doors. In contrast, meetings of the NIH Recombinant DNA Advisory Committee (RAC) were open to the public; moreover, streams of documents, minutes of meetings, internal memoranda and public letters flowed liberally from the committee. Bennett and his colleagues compensated for the lack of a complete published record by relying on interviews with key players in rDNA policy-making, including those involved in the Genetic Manipulation Advisory Group (GMAG). Their book provides a fine-grained analysis of the path towards the formulation of guidelines in Britain and their subsequent relaxation in response to changing perceptions of risk.

At the outset, American and British

biologists responded to the 1974 letter from Berg *et al.* according to the two nations' differing recent histories (the letter, published in both *Science* and *Nature*, urged that certain classes of rDNA experiments should be postponed until the risks were better understood). Many American scientists had been sensitized by the debates about atomic weapons and the role of "responsible physics" in a nuclear age, Robert Oppenheimer being the symbol of ambiguity. How can one be true to one's science, true to one's nation and true to one's humanity? When in doubt, which takes precedence? American involvement in Vietnam and the morally dubious roles of science and technology during the war were other factors shaping the consciousness of American scientists. Perhaps going public on gene splicing was the biologists' way of cleansing their consciences and setting themselves apart from the physicists and the chemists. British scientists, on the other hand, faced the rDNA issue in the wake of a widely publicized accident in which two people died after the escape of smallpox virus from a high-containment laboratory at the London School of Hygiene and Tropical Medicine. These backgrounds may explain some of the differences in how the respective political cultures handled rDNA policy-making.

In Britain, organized groups of teachers, civil servants, laboratory personnel and trade unionists were active in the policy process, while in the United States participation came from environmental groups and local communities. Compared to the United States, British rDNA policy was drawn up taking into account diverse interests, but it was all done in greater secrecy. *The Politics of Uncertainty* highlights differences in the way guidelines were established. British policy-makers sought a loose code, preferring general principles to detailed taxonomies of experiments and viewing the American system of strict guidelines as too inflexible. A canonical framework for assessing the potential risks of recombinant DNA experiments, introduced by Sydney Brenner, was an attempt to bring rationality and order to the taxonomy of cloning. When, in 1979, the approach was brought before the RAC for consideration, it was hastily dismissed. Not only did the committee fail to make sense out of it, but, more significantly, the scheme rated NIH containment levels for certain experiments as too low. Tightening up the rules on containment during a period when the US guidelines were on a fixed course of relaxation was too great a price for a dose of British rationalism.

In comparison to the British way of doing things, the American approach to rDNA regulation is legalistic and process-orientated. Typically, this reflects the

adversarial style of resolving environmental policy debates in the United States, where regulatory discretion is a target for litigation. The British regulatory system is more flexible, and thus, in the case of rDNA, was compatible with the adoption of general principles applied by scientific élites.

Notwithstanding these differences, the similarities in outcome were striking. Here were two countries which nourished highly advanced research communities in molecular genetics, which had captured a sizeable share of the Nobel prizes and which faced the prospect that strict regulation of rDNA research might delay the scientific progress of one relative to the other. Out of disparate policy cultures emerged unity of scientific values. The outcome was not coincidental but stemmed from the international and competitive nature of science. Both in Britain and the United States the passage of special legislation was avoided; the guidelines were viewed as an interim measure only, and subject to considerable relaxation; and a multi-tier review process for rDNA research activities was adopted. Even the taxonomies of potential risks varied little.

Overall, *The Politics of Uncertainty* emerges as the leading study of rDNA policy in Britain. The authors' insights about the blending of science and social process, the interplay of scientist and non-

scientist in risk analysis, and, more generally, the process of negotiating the control of research also make it a notable contribution to the broader literature of science policy.

Cloning and the Constitution arrives at a time when discussion about the rights and responsibilities of scientists has intensified. The US Office of Technology Assessment recently published *The Regulatory Environment for Science*, which examines issues of controls on research, and there are a number of cases where American communities have exercised control over research. On top of the furore over the release of genetically modified bacteria, the city of Cambridge, Massachusetts, recently banned the use of five chemical warfare agents in a testing programme funded by the military, and a farm-worker organization filed a suit against the University of California to stop its research on the mechanization of farming practices.

Carmen's book is made up of three elements: an answer to the question "Is scientific inquiry protected by the Constitution?"; an interpretation of selected periods of the rDNA controversy through a Constitutional perspective; and a report on Carmen's interviews with 52 individuals associated with rDNA research or its regulation. The unifying thread is the author's thesis on scientific inquiry and civil liberties, which is derived from his theory of a living Constitution. Because the US Supreme Court has not decided a case involving the rights of scientists to do research, this thesis is prescriptive only. It can be summarized as follows. Scientific inquiry falls on a continuum; on one end there is pure research, on the other, applied. Both types of research may involve experimentation. Pure research is a form of creative expression and deserves the status of quasi-speech. Once classified as quasi-speech, any research (including experimentation) should be afforded Constitutional guarantees under the First Amendment. To interfere in pure research, the government bears an "extraordinary burden of proof" that it needs to

do so. Because much of the early work involving gene splicing was part of basic research, from a Constitutional standpoint federal interference was unjustified.

Under this theory, applied research is treated as conduct or quasi-conduct and, as such, is not guaranteed protection under the First Amendment. For the cases between the extremes, the author suggests a "totality test" to ascertain whether the basic or the applied aspect of the research carries more weight.

Carmen, then, maintains that there is a fundamental right to scientific research (including experimentation) if the research is "pure"; but a rather more useful distinction has been drawn between cognitive/theoretical and experimental inquiry. Under this scheme, scientific work (whether basic or applied) that pertains to generating hypotheses, constructing explanations, propounding theories and the correlative activities of disseminating information, is protected unambiguously as a form of free speech. However, experimental work is a form of action, and it should not make a wit of difference, from the safety and ethical standpoint, whether the experimental programme is basic or applied.

Carmen has constructed a noteworthy thesis but one that I find caught in a web of ambiguity. The dichotomy between pure and applied research is not a sound basis for establishing Constitutional guarantees for science. Curiously, though, this concept has already found a place in the Department of Defense's (DoD) export control regulations on research results, part of a general policy designed to protect the national security and domestic economy of the United States. This goes back to 1984 when the US executive branch issued a draft policy justifying the application of export control regulations to basic research. The policy gave agencies of the government, including DoD, the right to censor any information that "can be used or adapted for use, in the design, production, manufacture, utilization, or reconstruction of articles or materials". In response to vigorous opposition among scientists to *post hoc* censorship of research results, DoD issued a policy clarification stating that export control regulations will not be applied to fundamental research unless that research has been initially classified. More than any other intervention into the scientific enterprise, national policies restricting the open communication of scientific results are the clearest infringement of civil liberties. Regrettably, these policies escape the critical eye of the author. □

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Skiping across the waterfront

Robert T. Rubin

Hormones and Human Behaviour. By Bernard T. Donovan. Cambridge University Press: 1985. Pp. 223. £25, \$39.50.

ON receiving *Hormones and Human Behaviour* for review, I was excited by the prospect that here was a succinct, integrated overview of "psychoneuroendocrinology", written with the consistency of a single author. Such a book would be of great value for students and others interested in acquiring a basic understanding of this complex field. I was further buoyed by the author's statement of purpose in the preface — we are told to expect an exciting, concise and intelligible review of present knowledge, not definitive or comprehensive, but a guide to the literature and of help to those working in the behavioural sciences or studying for a specialist qualification in psychological medicine. After careful reading of the contents, however, I fear that while the book contains a great deal of useful information, it falls short of the mark in several respects.

In writing the book, the author undertook a formidable task, perhaps an impossible one. In a mere 191 pages of text he has attempted to cover a vast range of topics: limbic/hypothalamic/pituitary/target endocrine gland interrelations; all the pituitary and other hormones; the roles of the many neurotransmitters and neuro-modulators in endocrine function; patterns of hormone secretion; cellular mechanisms of hormone action; the interrelations of hormones and brain development; and male and female sexual behaviour; aggression; control of food intake; emotion; and learning and memory. To cope successfully with all these subjects would require an economy of concept and of style quite beyond that shown here.

Clearly, Donovan has a broad interest in this field, and he has marshalled a mass of references into an impressive bibliography which is accurately cited in the text. However, he paints his subject with a broad, and occasionally reckless, brush. I should let some quotations speak for themselves: the hypothalamus "integrates almost all higher functions" (p.16); "hypothalamic function is modulated or regulated by the amygdala" (p.19); "The secretion of cortisol is increased in depressive illness" (p.25); the structural similarity between growth hormone and prolactin "gives rise to problems in hormone assay" (p.48); "it has become clear that the locus ceruleus... plays a major role in the control of anxiety" (p.169).

While each of these statements may be accurate when accompanied by the appropriate qualifiers, such qualifiers are too often absent or tacked on in desultory fashion at the ends of paragraphs.

In the book, Donovan covers the waterfront of psychoneuroendocrinology. However, the organization of the impressive array of facts he has brought together is often wanting, particularly in conveying the concepts and controversies surrounding behaviour-hormone interactions. The introduction and overview, which, conceptually, ought to be the clearest of the chapters, is a jumble. By contrast the paragraphs on homosexuality (pp.129 and 130) are well-balanced. If only the rest of this volume were consistently so.

In his final chapter, "What Next?", Donovan puts forth some cautions and caveats which he himself has failed to observe earlier in the book. For example: "It is not enough to refer to 'depression' or 'schizophrenia.'" The endocrine evidence points to different categories or groups of

depressed or schizophrenic patients" (p.188). He further states, "Hormonal disturbances seem to be less common in psychiatric patients than the incautious reader of Chapter 1 might be led to expect" (p.188). We well may ask, especially of a volume intended for beginners, who should be more cautious, the reader or the author?

The subject matter of this book fits in well with the aims of the series, *The Scientific Basis of Psychiatry*, of which it is part. The book also highlights an area of the "mind-body interface" that is growing ever more important (and ever more complex), and it contains an abundance of fascinating material. But it should be read with great caution, especially by the naive reader hoping to find a trustworthy introduction to psychoneuroendocrinology. □

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Zeolitic first

William S. Wise

Natural Zeolites. By Glauco Gottardi and Ermanno Galli. Springer-Verlag: 1985. Pp. 409. DM 160.

BY various counts, depending on nomenclature and definitions, there are at least 42 different zeolite minerals, some of which have been known since the eighteenth century. In *Natural Zeolites* Gottardi and Galli have provided the first book entirely devoted to the description of them. Writing it must have been difficult, because new physical descriptions and fresh information on occurrences are continually accruing. But, for the moment at least, this book is a valuable addition to the literature.

In an account of this nature the topics that should be included are some sort of classification, and for each species a description of crystallography (morphology and crystal structure), physical properties, chemical compositions (including dehydration and ion exchange data), occurrences (localities and geology), and nomenclature and history. All of these are covered to some extent. As Gottardi and Galli note in their preface, however, no book could satisfy all readers in the completeness of its coverage. Because the authors are structural crystallographers, the emphasis of the book is appropriately upon a new structural classification and upon details of framework geometries, such as ordering.

Certainly, the topic of widest interest beyond mineralogy is the framework arrangements of the various minerals or

groups of minerals. In the introductory chapter, these structures are well described and illustrated not with the usual stereographic drawings, but with "tetra-units and spaghetti", a technique developed by Gottardi himself. I found these drawings to be a particularly effective way of depicting most of the three-dimensional arrangements. Other strengths of the book are the detailed comments on the historical development of nomenclature, the critical evaluation of structural crystallography, and the inclusion of newly determined DTA, TG, DTG curves and new x-ray powder diffraction data for most species.

My only disappointment was with the depth of treatment of the occurrences and of paragenesis. For the most part, in this book zeolites are classified as "sedimentary" or "hydrothermal". Actually the first category is the diagenetic alteration of volcanic glass in sediments, while the second pertains to the large crystals found mostly in cavities of volcanic rocks. To be sure, many of these are results of low-temperature (<150°C) hydrothermal alteration of volcanic piles. However, there are also large areas of zeolite occurrence where these minerals grow as a result of diagenetic adjustment of the basalt or andesite to shallow burial and saturation with ground water. □

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• Newly published by Elsevier is *Zeolites: Synthesis, Structure, Technology and Application*, the proceedings of a symposium held by the International Zeolite Association in Yugoslavia in 1984. Editors are B. Držaj, S. Hočevar and S. Pejovnik, price is Dfl. 345, \$127.75.

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Bent for philosophy

H. C. Longuet-Higgins

Mind from Matter? An Essay on Evolutionary Epistemology. By Max Delbrück. Edited by Gunther S. Stent *et al.* Blackwell Scientific: 1986. Pp.290. Hbk £29.50, \$29.95; pbk £14.80, \$14.95.

MAX Delbrück was one of the very few physicists who have ever really made good in biology. A not unusual pattern is for an eminent physicist to distil into a public lecture or a slim volume his or her philosophical reflections on the nature of life and then to rest on his Laureate, leaving younger scientists to fill in the essential details. Fortunately for molecular biology, Delbrück never achieved great distinction in theoretical physics, and wisely decided, in the mid 1930s, to devote himself to the study of bacterial viruses and, later, bacterial resistance to virus infection. In 1969, with Luria and Hershey, he won the Nobel Prize in Medicine or Physiology. But all his life Delbrück was an earnest philosopher of science, having sat as a young man at the feet of Niels Bohr and heard him discourse upon the principle of complementarity and how it might apply to living organisms. Delbrück's 1949 essay *A Physicist Looks at Biology* was his first — somewhat baffling — contribution to the philosophy of science; his posthumous book *Mind from Matter?* he epitomized as "an investigation into human cognitive capabilities, as expressed in various sciences".

Mind from Matter? is a labour of love by a number of Delbrück's friends — most notably Gunther Stent, whose introduction to the book is a model of clarity and insight. Stent evidently shares Delbrück's perplexities about the relations between life and non-life, mind and matter, and appearance and reality, and this makes it possible for him to act as a sympathetic interpreter and expositor of Delbrück's often difficult ideas. As he explains, the book originated as a course of lectures at Caltech on "evolutionary epistemology" — a phrase strongly reminiscent of Jean Piaget's "genetic epistemology", which Delbrück freely acknowledges as a major philosophical influence. Unfortunately, Delbrück's declining health did not permit him to do more than publish a couple of articles summarizing the lectures; the present volume is consequently a heavily restored mosaic of Delbrück's notes and the lectures he based on them, recorded and interpreted by his students and colleagues. It would obviously be ungenerous to criticize too sharply the outcome of their unselfish endeavours.

Delbrück's message is not easy to capture in a few sentences, because the book ends by declaring its own title to be

absurd, claiming with no obvious justification that "if we can learn to accept this ultimate absurdity, there may yet be hope for developing a formal approach that will permit a grand synthesis". But it is better to travel hopefully than to arrive, and *Mind from Matter?* supplies much intellectual refreshment along the road. The first half-dozen or so chapters present a matter-of-fact, almost tedious account of cosmic evolution, the origin of life and the ascent of man. Then there is a subtle change of perspective and we find ourselves looking at perceptual and cognitive psychology *from the inside*. In discussing the acquisition of fundamental physical concepts Delbrück leans heavily on the writings of Piaget, and reveals him in his true colours as a philosopher of mind rather than a mere developmental psychologist.

There follows a set of chapters on pure mathematics and the paradoxes which gave birth to modern mathematical logic. Delbrück makes the interesting proposal that our mathematics inevitably suffers from the same limitations as our own minds when we attempt to describe those parts of reality which lie too far outside our day-to-day experience. Delbrück obviously enjoyed airing his wide knowledge of number theory and quantum theory, but all too often the intellectual virtuosity of the book obscures its main theme. Relativity is used as a text for the unreliability of naive physics; quantum theory as a warning against attaching too much objectivity to the outcome of a measurement. The chapter on "the cartesian cut" argues cogently that many of our most acute philosophical problems arise from the misguided attempt to erect a barrier between subjective and objective reality: "Is there not but one reality: the act of seeing what our language makes us call an 'object'?"

Unfortunately the chapter on language is something of a disappointment when it arrives; it is largely taken up, in fact, with a cursory account of artificial intelligence. Perhaps Delbrück felt that AI raises philosophical issues which he hadn't yet had time to think about, but must nevertheless be raised in any serious discussion of the nature of mind.

What the professional philosophers will make of *Mind from Matter?* remains to be seen, but at least it should be required reading for students of the philosophy of science, who so often pronounce on the notions that inform scientific enquiry without acquainting themselves with the considered philosophical positions of real scientists. Max Delbrück may not have been a great philosopher, but he certainly knew his trade as a scientist. □

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Milankovitch climatic origin of mid-Cretaceous black shale rhythms in central Italy

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The Earth's orbital variations, reflected in Pleistocene ice volume, were also recorded in non-glacial times. Carbonate production in pelagic mid-Cretaceous sediments, quantified by calcium carbonate and optical densitometry time series, reflects the orbital eccentricity and precessional cycles. Minimal eccentricity brought deep-sea anoxia. Most of the sedimentary variability of this 100-Myr-old sequence lies within the Milankovitch (orbital) frequency band.

TIME-SERIES analysis of several parameters of deep-sea sediments (carbonate content¹⁻³, accumulation rate of aeolian material⁴ and oxygen isotope values in carbonates⁵⁻⁷) has provided measures of climatic variability during the past two million years, and has demonstrated its sensitivity to periodic changes in the geometry of the Earth's orbit⁷⁻¹⁰. Several studies¹¹⁻¹⁷ have found sedimentary rhythms in ancient sediments at periods approximating those of the modern orbital parameters, suggesting that Milankovitch (orbitally induced) climatic rhythms have persisted at their modern frequencies throughout a large portion of the geological record. This conclusion raises the possibility of a comparative study of the climate system's response to a continuing series of perturbations under different boundary conditions (continental positions, sea level, extent of continental glaciation, oceanic interconnections and atmospheric CO₂ levels). Most studies of the pre-Pleistocene have relied on somewhat subjective field measures of cyclicity, such as thickness of strata; very few sets of finely sampled, objective measurements allow for a closer comparison with the modern record of astronomical variations. We report here high-resolution data on oxidation-reduction (redox) conditions and carbonate cyclicity during an estimated 1.6 Myr of deep-sea history from the mid-Cretaceous (100 Myr BP) of central Italy, in which the character of the cycles, as well as their estimated periods, clearly link the variability to Milankovitch precessional and eccentricity forcing. The dominance of 100-kyr and 400-kyr cyclic components during a non-glacial era raises questions about the present understanding of climatic change.

Numerous data indicate that Cretaceous climate was significantly different from the present. Oxygen isotope studies imply deep-water temperatures 10–12 °C warmer than at present^{18,19}. Large-scale continental glaciation was absent and climate was more equable²⁰⁻²². The warmer global Cretaceous climates seem to require a CO₂-rich atmosphere^{21,23}; thus, studies of mid-Cretaceous climate dynamics may furnish a crude analogue to climates of the anthropogenic greenhouse of the future. The questions we shall explore are: what is the evidence of climate change on a thousand- to million-year timescale during the Cretaceous, how does this variability compare with what is known from the Pleistocene climate record, and what mechanisms drove Cretaceous climate oscillations?

The interval analysed comes from part of a core 84 m deep cut through the Scisti a Fucoidi (Fucoiid Marls) in Piobbico (Marche), in the central Apennines of Italy, as a joint project by the US National Science Foundation and the Italian Consiglio Nazionale della Ricerche. The core spans most of the Aptian

and Albian stages of the Cretaceous and consists of deep-sea foraminiferal-coccolith limestones and marls deposited on the southern side of the narrow Tethyan seaway at a palaeolatitude of ~19° N (ref. 24). The lithology shows spectacular ranges in oxidation state, with intervals of highly oxidized (red), intermediate (white to drab) and highly reduced (black) strata. The mixing of one lithology into another by burrowing organisms indicates that the alternations are of primary origin. Frequent black, finely laminated beds, 2–20 cm thick and containing 1–9% organic carbon, record episodes of seafloor anoxia in a pattern similar to that found in other mid-Cretaceous ocean basins^{13,25-28}. The suite of microfacies within black shale bands—a dark, burrow-mottled base, an intermediate black, but unlaminated portion and an upper laminated interval—indicates that these deposits are not turbiditic, and record *in situ* dysaerobic to anaerobic conditions.

Cyclic sedimentation

Outcrop studies of bundled portions of the Umbrian sequence (Barremian¹⁶, Albian-early Cenomanian¹⁴ and late Cenomanian¹⁶) have led to the suggestions that the bedding couplets reflect the Earth's axial precession cycle (mean period 21 kyr) and that the bundles mirror the short cycle of eccentricity (~100 kyr). In the Piobbico core, a ~8.7-m-thick interval from the upper Albian foraminiferal zone of *Ticinella breggiensis* (subzone *T. praeticinensis*) (I. Premoli Silva, personal communication) was chosen for detailed analysis. Because the core intersected strata dipping at 23°, the data to be presented are plotted against true thickness rather than core thickness. Our initial observations indicated that the sequence represents a repetitive alternation of more calcareous, relatively oxidized beds with more shaly, reduced beds. Stratigraphic variations in this interval were quantified using two techniques. Calcium carbonate content (Fig. 1a) was measured by a Coulometrics Inc. coulometer at ~2-cm spacing. Variations in darkness of colour were determined by densitometry: scaled and colour-corrected 35-mm diapositives ('slides') of split-core sections were measured for light transmission in a Perkin-Elmer microdensitometer at a sampling rate of 50 data points per cm of core length, and 25 parallel passes per slide were averaged along bedding and compiled into the curve shown in Fig. 1b. Values of transmission are low in dark beds and high in light beds, thus the curve yields a measure of the oxidation state of the sediment. The analysis was discontinued when the core colour changed from drab to dominantly red at 7.5 m depth in the section.

One way of approaching the question of rhythmicity is to

identify the cycles by simple inspection, and to estimate their duration from sedimentation rates; an alternative approach involves spectral analysis. As will be shown, the two approaches yield slightly different results.

A back-to-back or mirror plot of the curves (Fig. 1) expands where carbonate values are high and colour is light, and contracts for lower carbonate values and darker colours. The high-frequency oscillation depicts the bedding couplets, and the curve is segmented by an oscillation of lower frequency: typically two or three couplets with a comparatively carbonate-rich and drab-coloured marl member are succeeded by two to three couplets having marl members of lower carbonate content and darker colour, commonly black and laminated. This segmentation of the mirror-plot is the bundling of couplets so evident to the eye in outcrop. For reference the bundles have been assigned letters (Fig. 1). In addition, the envelope of the mirror plot (dotted line) shows the presence of a longer cycle.

The 8 m length of core contains 74 ± 4 couplets and 17 ± 0.5 bundles (the bundles become poorly defined near the top of the record). Statistics for mean thickness are provided in Table 1. We postulate that these couplets and bundles are the products of rhythmic forcing functions. If so, their periods can be derived from the mean sedimentation rate. The length of the Albian age is variously estimated at 15.5, 12 and 11.5 Myr (refs 29, 30 and 31, respectively); the 65 m of Albian at Piobbico (I. Premoli Silva, personal communication) therefore accumulated at a mean rate (compacted) of $5.0 \pm 0.75 \text{ m Myr}^{-1}$, yielding a couplet period of 21–22 kyr and a bundle period of 95–96 kyr (Table 1). The long cycle, which acts as the amplitude-modulating frequency of the bundle cycle, is depicted by the envelope of the mirror curve, and has an apparent wavelength of $\sim 2 \text{ m}$, corresponding to a period of 400 kyr. The three frequencies found thus yield close matches to the means of the present axial precession, the short cycle of orbital eccentricity, and the long eccentricity cycle¹⁰. Table 1 includes estimated periodicities from

Table 1 Lower Cretaceous carbonate cycles in the Umbrian region

	Thickness (cm)	Duration (kyr)
Late Albian, Piobbico (65 m)*		
Couplet	10.8	$21.6 \pm 20\%$
Bundle	48	$96 \pm 20\%$
Long cycle	200	$400 \pm 20\%$
Barremian ¹⁶ (80 m)*		
Couplet	28.3	$19 \pm 50\%$
Bundle	130	$88 \pm 50\%$
Late Cenomanian ¹⁶ (36 m)*		
Couplet	21.7	$29 \pm 25\%$
Bundle	106	$141 \pm 25\%$

Sedimentation rates calculated using refs 29–31, 59.

* Thickness of stage.

ref. 16, derived from other portions of the mid-Cretaceous Umbrian sequence. The consistency of the estimates over $\sim 25 \text{ Myr}$, despite uncertainties in the dating of Cretaceous stages and errors introduced by the use of mean sedimentation rates, argues strongly for the persistence of frequencies of 20–25 kyr and 100 kyr.

The 8-m core segment discussed here appears to record $\sim 1,600 \text{ kyr}$ of history. Figure 2 shows a comparison of the 7.2-m carbonate curve with 1,500 kyr of orbital eccentricity variations, calculated from ref. 9 and extending from 1,350 kyr BP to 150 kyr AP (the comparison is intended to be qualitative; no match of absolute values is intended). The core data (Fig. 2a) are shown as a smoothed curve obtained by a cubic spline routine, in which the precessional signal and possible high-frequency noise have been eliminated. Curve *a* in Fig. 2 matches the orbital signal (Fig. 2b) in number of elements and shows similar changes in

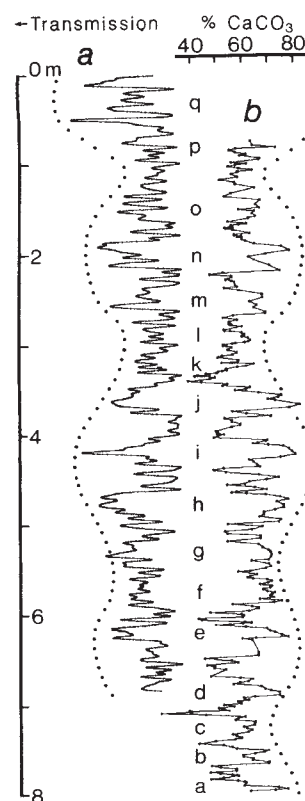


Fig. 1 Transmission (*a*) and carbonate content (*b*) versus thickness in metres for part of Upper Albian section of core (*T. praeticinensis* foraminiferal subzone). Transmission curve is plotted to mirror carbonate data: values of transmission (brightness) increase towards the left, while dark beds plot to the right. Dots indicate carbonate sample points. Individual bundle cycles are lettered.

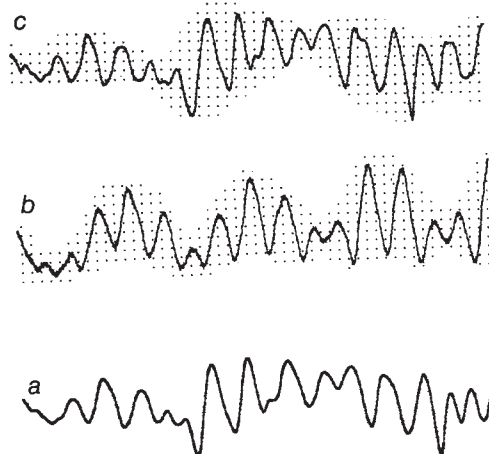
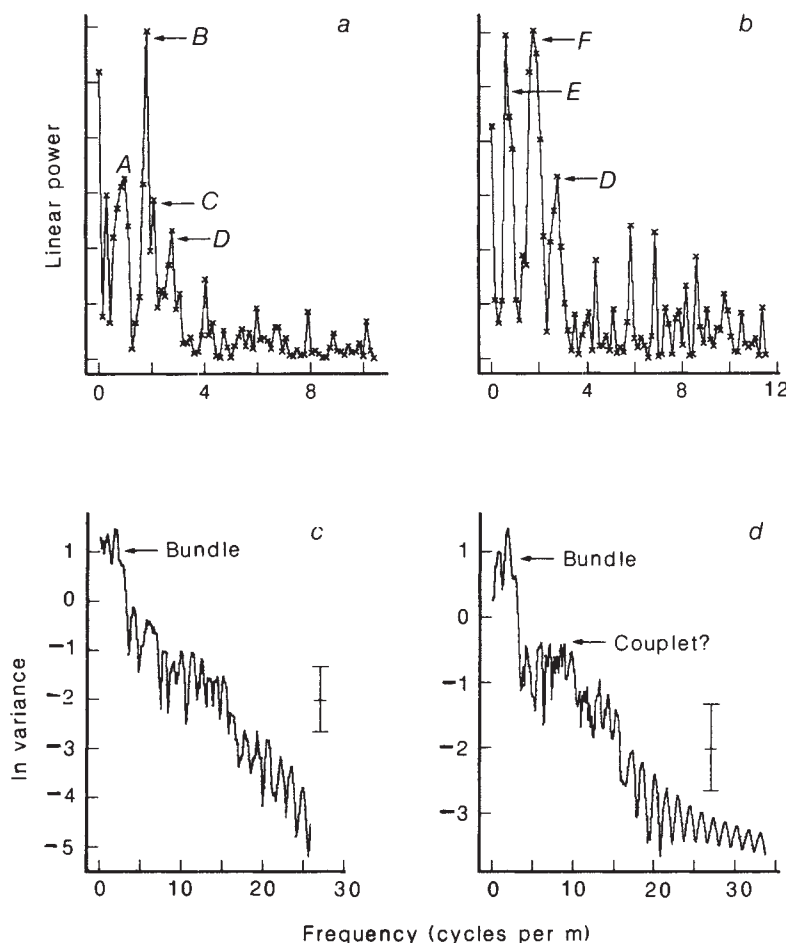


Fig. 2 Piobbico carbonate series (*a*), smoothed by a cubic spline routine, compared with the orbital eccentricity curve⁹ (*b*) for the period 1,350 kyr BP to 150 kyr AP. A simple tuning of the carbonate record to constant spacing of peaks and troughs (*c*) results in a good match to the orbital curve (*b*). Stippled pattern emphasizes beating pattern of both records, with nodes of low amplitude coinciding with minima of the 413-kyr eccentricity term in the orbital series.

Fig. 3 *a*, Detail of carbonate power spectrum, showing peaks at: *A*, 90 cm (225 kyr); *B*, 53 cm (108 kyr); *C*, 48 cm (98 kyr); and *D*, 36 cm (73 kyr). *b*, Detail of transmission power spectrum, showing peaks at: *E*, 171 cm (347 kyr); *F*, 57 cm (116 kyr); and *D* (as in *a*). In *a* and *b*, higher-frequency peaks, probably harmonics of the ~100-kyr cycle, are not labelled. *c*, *d*, Lower-resolution power spectrum of carbonate (*c*) and transmission (*d*) curves plotted as $\ln$ variance. 90% confidence interval and bandwidth (vertical and horizontal error bars) calculated following ref. 60.



amplitude, which waxes and wanes with a 2-m wavelength. However, the peaks and troughs of these curves are not well aligned. The length of bundles (see Fig. 1) varies by a factor of two. If the bundles are of equal duration, then their sedimentation rate has also varied by the same factor, and the stratigraphic dimension departs strongly from time. The fact that the couplet periodicity also narrows during short bundles and lengthens during long ones indicates a coherent shift in apparent frequency which is best explained by changing sedimentation rates. The carbonate-poor bundles, showing low amplitude in Fig. 2*a*, are the short members. A simple tuning which assumes that maxima and minima of the carbonate bundles demarcate approximately constant time increments, and assignment of a periodicity equal to the modern mean value of 95 kyr (compare the 95–96 kyr estimated from the mean sedimentation rate) results in a good match between the core-derived curve (Fig. 2*c*) and the modern orbital cycle (Fig. 2*b*).

Departures from ideal cyclicity shed light on the dynamics of Tethyan deep-sea sedimentation. We consider the record to be the product of forcing functions, periodic in time but stratigraphically distorted by variable sedimentation rate. These distortions are to a large degree a function of the cyclicity itself: where mean carbonate values are high, the redox (bundle) cycle is stretched; where low, it is contracted. This is not a reflection of variable compaction: densities in fact increase with carbonate content, from $\sim 2.45 \text{ g cm}^{-3}$ in very marly beds to 2.65 g cm^{-3} in the most limy beds. Analyses in progress indicate that the total clay content per bundle shows much less variability than does carbonate content. Thus, carbonate supply seems to have been the chief variable in sedimentation rate.

Good to excellent preservation of foraminifera suggests little

or no seafloor dissolution of calcite and identifies the cycle as one of biogenic carbonate productivity. Black marls, indicative of a dysaerobic to anaerobic sea floor, correspond to episodes of low carbonate productivity. The very modest rates of organic carbon accumulation during anoxic events in the Atlantic and Tethys^{14,32} are consistent with the picture of lowered surface fertility that emerges from the Piobbico time series. At the couplet level, variations in carbonate content (31–84%) suggest variations in productivity of up to a factor of 9. The rate of clay supply may have varied at this level, and the couplets may have been enhanced by diagenetic solution transfer of carbonate from marls to limestones³³, but carbonate productivity still appears to be the most variable factor, controlling sedimentation rate in response to orbitally induced climatic change.

Spectral analysis

Figure 3 shows power spectra derived from the carbonate content and transmission time series in Fig. 1. Figure 3*a* (carbonate) and *b* (transmission) show details of the spectra in the frequency range discussed above (wavelengths of 4 m to 10 cm), whereas in Fig. 3*c* (carbonate) and *d* (transmission) the spectra are plotted as the natural log of the variance, and smoothed with a five-point moving average. The frequency range is extended to 25 (Fig. 3*c*) and 30 (*d*) or to ~8- and 7-kyr periods. The detailed spectra show the following large peaks: for the carbonate content at about 90 cm (225 kyr), 53 cm (108 kyr), 48 cm (98 kyr) and 36 cm (73 kyr); for transmission at about 171 cm (347 kyr), 57 cm (116 kyr) and 36 cm (73 kyr).

The low-frequency terms correspond quite well to the estimated periodicities of elements of the present eccentricity cycle. Although resolution is low at this end of the spectrum, the

171-cm peak (*E*, Fig. 3*b*) in the transmission series seems to correspond to the 2-m wavelength visible to the eye. The presence of terms at slightly greater and less than 100 kyr (Fig. 3*a*, *b*, peaks *B–D*, *F*) suggests peak splitting of a ~95-kyr periodicity, and corresponds to the amplitude modulation of the bundle cycle by the 2-m (400-kyr) cycle. An unexplained component is the carbonate peak at ~225 kyr (peak *A*, Fig. 3*a*). At higher frequencies, most peaks seem to correspond to harmonics of the bundle frequency.

The low-resolution spectra (Fig. 3*c*, *d*) demonstrate that virtually all of the sedimentary variance lies within the Milankovitch frequency band (periods ≥ 12 kyr). There is a general resemblance to the Pleistocene $\delta^{18}\text{O}$ spectrum^{7–8} in that most of the amplitude is concentrated in a peak near 100 kyr; however, the peaks in the 11–65-kyr range are very weak. The transmission spectrum (Fig. 3(*d*)) shows a broad band of power in the range 8–10 cycles per m range which may match the couplet frequency, but most of the spectrum in this region is dominated by harmonics of the bundle peak. This is to be expected from the sedimentational distortion of the time axis discussed above: variations in the spacing of the bundle cycle, as well as systematic shifts in accumulation rate with the cycle phase (lower accumulation in the troughs) will generate higher-frequency multiples of the dominant peak. The even higher variation in sedimentation rates at the precessional level (21–22 kyr), compounded by the inherent range in the precession period¹⁰, masks the high frequencies. A detailed statistical analysis of the series broken into shorter time windows is in preparation (T.D.H. and J. Park), and shows promise of yielding an objective tuning function to compensate for some of the distortion introduced by variable sedimentation rate.

Cretaceous climate dynamics

Our data have important implications for mid-Cretaceous climatology and the origin of the black shales which were so common during this time. The mid-Cretaceous has been considered to be a time of generally 'sluggish' oceanic and atmospheric circulation due to low Equator-to-pole temperature gradients^{21,22}, with moribund overturning of the water column leading to the common occurrence of black shales^{25,32,34}. The high-frequency excursions in deep-water oxygenation and surface productivity recorded in the Piobbico core indicate that stagnation occurred only episodically and do not imply continuous slow circulation. The prominence of carbonate and redox cyclicity suggests a highly dynamic ocean–climate system, in which wide swings in climatic regime occurred in response to external forcing; there is no evidence of sluggishness on a thousand-year timescale. If carbonate cycles are indeed reflective of Tethyan surface fertility, then large changes in deep-water upwelling rate must have occurred on a geologically rapid timescale.

The lack of spectral amplitude at the 41-kyr obliquity frequency indicates that our data record predominantly low- to mid-latitude climatic effects. Theoretical and empirical work indicates that low-latitude regions are sensitive to the precessional and eccentricity parameters, which control seasonality and intensity of monsoonal circulation^{35–37}. Of several climatic mechanisms that might link orbital forcing to oceanic anoxia, perhaps salinity and temperature variations are the most plausible. In a salinity-driven model, abrupt freshening of the surface layer leads to reduced vertical mixing and alters thermohaline exchange of water between ocean basins. These factors are crucial to the nutrient and oxygen balance of an ocean basin, and hence to production of biogenic material and preservation of organic matter. The correlation between orbital cycles, monsoonal intensity and anoxic events in the Pleistocene eastern Mediterranean has been convincingly demonstrated by Rossignol-Strick³⁵. Recent model simulations of Cretaceous climate have in fact predicted sensitivity of the monsoonal response along the margins of the Tethys to precessional forcing³⁸.

Weissert *et al.*³⁹ have used palynological data from the Lower Cretaceous of Italy to correlate the onset of black shale rhythms with a shift to a more humid climate.

Climatic modulation of deep-water temperatures might also explain the cyclic occurrence of black shales. Brass *et al.*⁴⁰ have postulated that intrusions of warm, saline bottom water, capable of holding less oxygen than the normal cooler bottom water, may have episodically pushed already poorly oxygenated Cretaceous deep water over the threshold to anoxia.

Individual anoxic episodes in the Piobbico sequence occur with a 20-kyr period, as did the Pleistocene anoxic events of the eastern Mediterranean. However, the pattern in the Cretaceous seems to have been anti-monsoonal: anoxic events took place during periods of low amplitude of the eccentricity and precessional cycles. In order to match the phases of carbonate and redox cycles with an eccentricity input function, we examine the systematic changes in amplitude of the bundle cycle. Association of high carbonate content and lighter colour with maxima of orbital eccentricity is possible by matching the characteristic nodal pattern of the Berger eccentricity curve⁹ to the similar pattern in the Piobbico records. Computation of eccentricity for the past 5 Myr shows that the node of minimum 100-kyr amplitude stays locked into phase with the minima in the 413-kyr term. A decrease in amplitude is also associated with a shift to slightly higher frequency. Minimum-amplitude fluctuations in the Piobbico time series occur during periods of generally low carbonate and oxidation state (for example, bundles *l* and *k*, *p* and *o*). If the eccentricity phase relationships can be extrapolated into the past, the lows of carbonate and transmission values follow lows in eccentricity. Individual anoxic episodes would then follow precessional minima (lowered monsoonal intensity) during periods of generally low eccentricity.

We prefer to correlate Tethyan anoxic events with periods of minimal seasonal contrast because of the above-mentioned phase match, and because such a mechanism could lead to a relatively simple covariance of surface productivity and deep-water oxygenation. Modulation of seasonal temperature contrast by orbital cycles could be expected to influence thermocline stability, wind stress and rates of deep water formation, resulting in changes in flux of nutrient-rich deep water to the surface over time. For example, Prell³⁷ has related the record of upwelling intensity off the Arabian coast with the strength of the Northern Hemisphere precessional cycle. We interpret variability in carbonate in our sequence as reflecting climatic control on the rate of upwelling of deep water to the surface ocean. In our model, black shale events occurred as rates of deep water formation slowed due to lessened seasonality. If deep water was formed at the coldest time of the year, then periods of minimal seasonal contrast would produce warmer, less oxygenated bottom water than periods of precessional highs. Anoxia would therefore be linked to lower overturning rates and lowered carbonate productivity.

Implications

The strong eccentricity and precessional power in the Tethyan region indicate that changes in seasonal contrast may leave a distinctive mark in the geological record. The striking similarities of the Piobbico Aptian–Albian sequence with early- to mid-Cretaceous rhythmic deposits in other ocean basins (see, for example, refs 26–28, 33, 41) leads us to expect that time-series analysis of other records will confirm the global signature of climatic rhythms in the mid-Cretaceous deep-sea record. A number of workers consider many of the deep-water black shales to be turbiditic in origin, or unreflective of widespread deep-basin anoxia^{42–45}. Detailed sampling such as that reported here may help to establish whether these sequences record external climatic forcing or random local events.

Quantitative understanding of the translation from insolation cycles to palaeoceanographic response remains a major chal-

lenge. Recent theoretical work on climate change during the Quaternary has emphasized the importance of internal mechanisms, associated with ice sheet dynamics, which might enhance what are considered to be the rather weak primary Milankovitch signals^{46,47}. Various feedback mechanisms linked to glaciation, such as ice-albedo effects, bedrock response to glacial loading, and the hydrological cycle, seem capable of generating large global temperature and ice volume changes when forced by orbital variations⁴⁸⁻⁵⁰. The hypothesis of sluggish Cretaceous circulation has also been based on the supposition of the centrality of polar conditions to world climate dynamics^{22,34}. Our data suggest that ice sheet amplification may not be a prerequisite to large-scale climate modulation by cyclic variations in insolation. The carbonate content variations in the interval sampled are comparable to glacial/interglacial fluctuations observed in Pleistocene deep-sea cores; similarly, the high-amplitude redox cycles in our core, and in similar Cretaceous sequences in the North and South Atlantic, argue for significant climatic-oceanographic change on a timescale of 10-100 kyr. These changes were apparently driven by low-latitude (eccentricity and precessional) forcing.

Another intriguing observation is the prominence of the 100-kyr cycle, with its modulation at slightly more than 400 kyr, in the Piobbico core. Although calculated eccentricity variations should not have a significant effect on mean annual insolation^{9,51}, 100- and 400-kyr peaks are in fact large spectral components of the Pleistocene ice volume^{5,6,8}, pelagic carbonate^{1-3,52,53} and magnetic intensity⁵¹ records. The origin of these spectral peaks is the subject of controversy^{6,46,48-50,53-57}. Although many authors have ascribed the 100-kyr period to nonlinearities associated with ice sheet growth and decay^{48-50,54,55}, 100- and 400-kyr cycles appear to have controlled a large part of the variance in this deep-sea record of the non-glaciated Cretaceous. Several interpretations are possible. Nonlinearities not associated with ice may play, or have played during the Cretaceous, an important part in climatic response

to orbital forcing. The 100- and 400-kyr frequencies may thus result from the climate system's nonlinear response to the precessional signal rather than from a direct effect of the 100- and 400-kyr insolation cycles^{46,54,57}. Alternatively, evidence from the Pleistocene appears to indicate that eccentricity forcing exerts a major direct control on climate⁸. The strong resemblance of our Cretaceous time series to a linear combination of the precessional and eccentricity cycles reinforces this impression.

The redox and carbonate cyclicity exhibited in our core section appears to have been highly stable over time. The estimated 100-kyr bundling can be followed visually in photographs of core sections for >50 m, or 10 Myr. In addition, outcrop studies^{14,16} of the formations above and below the Piobbico core section show the persistence of these periodicities. This stability contrasts sharply with the changes in power of the Milankovitch frequencies in the Pleistocene ice volume record, in particular the 100-kyr cycle⁵⁸. Abrupt shifts in the character of the $\delta^{18}\text{O}$ record from earlier higher-frequency to dominant 100-kyr periodicity at ~700 kyr BP have been postulated to be the effects of discontinuous ice-cap sensitivity to small changes in insolation⁵⁴. The absence of glaciation in the mid-Cretaceous may simplify study of the climate system, in that it removes a potentially large source of internal variability, and account for the regularity of the 100-kyr cycle in our records. Palaeoclimatic studies of the non-glacial past may thus prove to be useful in testing theories of Pleistocene climatic change and in separating glacial effects from other responses of the Earth's climate to orbitally modulated variations in insolation.

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High frequencies of α -thalassaemia are the result of natural selection by malaria

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The frequency of α^+ -thalassaemia, but not other unlinked DNA polymorphisms, exhibits an altitude- and latitude-dependent correlation with malaria endemicity throughout Melanesia, supporting the hypothesis that protection against this parasitic disease is the major factor responsible for the high frequencies of haemoglobinopathies in many parts of the world.

THE haemoglobinopathies have provided one of the few convincing demonstrations of selection influencing single-gene frequencies in human populations. The high carrier rates for lethal disorders such as sickle cell anaemia and β -thalassaemia, which occur almost exclusively in those tropical and subtropical areas lying within the 'malaria belt', led Haldane¹ to propose that malaria might be the selective agent responsible, balancing loss of thalassaemia or sickle genes by premature death in homozygotes with increased fitness of heterozygotes in the malarial environment. Epidemiological and *in vitro* studies on sickle cell anaemia subsequently provided strong support for this hypothesis, and there is circumstantial evidence that high frequencies of β -thalassaemia may also result from malarial selection²⁻⁷.

Although α -thalassaemia is found throughout the malarial regions of the world^{7,8}, and might therefore be expected to conform to the pattern established for β -thalassaemia and sickle cell disease, there has been little evidence that malarial selection is responsible for the observed distribution of α -thalassaemia. This reflects important differences between the α - and β -globin gene disorders. As there is only one β -globin gene per haploid genome, the phenotypic effect of severe mutations at this locus is such that heterozygous carriers can be identified unequivocally. On the other hand, normal individuals have two α -globin genes per haploid genome. Deletions of a single α gene result in α^+ -thalassaemia⁸. Homozygotes for this condition possess two α genes ($-\alpha/-\alpha$) and heterozygotes three α genes ($-\alpha/\alpha\alpha$). The former condition is characterized by a mild hypochromic anaemia but the latter is often indistinguishable from normal. Deletion of the linked pair of α -globin genes results in α^0 -thalassaemia. Homozygotes ($--/--$) have no α -globin genes and are stillborn; heterozygotes ($--/\alpha\alpha$) have a mild hypochromic anaemia indistinguishable from homozygous α^+ -thalassaemia; the phenotypic effect of the loss of two α -globin genes is the same regardless of whether they are missing from the same ($--/\alpha\alpha$) or opposite pairs ($-\alpha/-\alpha$) of chromosomes. Thus the genetics of the α -thalassaemias is more complex than that of the β -globin disorders, and while there is a good overall correlation between the changes in red cell morphology,

reduced haemoglobin content and α -globin synthesis, these parameters are often of little value in distinguishing between genotypes⁹. Hence, until recently it has been extremely difficult to study the population genetics of α -thalassaemia.

The introduction of DNA analysis has made possible the unequivocal diagnosis of the carrier states for most forms of α -thalassaemia¹⁰. With this has come the realization that the disease occurs at extremely high frequencies in many populations; indeed it now appears that it is the commonest single-gene disorder in the world⁸. In addition, accurate assignment of genotypes by gene mapping has at last allowed possible relationships with malaria to be investigated.

We have used this approach to study the distribution of α -thalassaemia and its relationship to malaria in the South-West Pacific (Fig. 1). These island populations offer several advantages for this type of study. Malaria endemicity varies in two dimensions, with altitude in Papua New Guinea and with latitude in Island Melanesia^{11,12}. This situation provides the opportunity to compare the frequencies of α -thalassaemia in Melanesians exposed to different malaria endemicities. Furthermore, the β -globin chain variants Hb S and Hb E, which are probably subject to stronger selection and hence might complicate the analysis of any relationship between the distribution of malaria and α -thalassaemia, are not found in Melanesia⁷. Similarly, β -thalassaemia, which has a more severe phenotype, is absent or uncommon on most of the islands¹³ and our preliminary surveys have shown that α^0 -thalassaemia ($--$), due to deletion of the linked pair of α genes, which is found in South-East Asia and the Mediterranean⁸, is very rare or absent in Melanesians¹³. Because of the suggested competitive interaction in a population between the single ($-\alpha$) and double α -gene ($--$) deletions¹⁴, a significant prevalence of the latter might well have obscured any frequency correlation between the common single-gene deletion forms of α^+ -thalassaemia and malaria. Finally, although the present endemicities of malaria in this region may have been altered by control programmes^{15,16}, numerous malaria surveys were performed before such intervention began^{11,15,17-31}.

We have compared the endemicity of malaria recorded in these early surveys with the frequencies of α -thalassaemia determined by analysis of over 1,800 DNA samples. We find that

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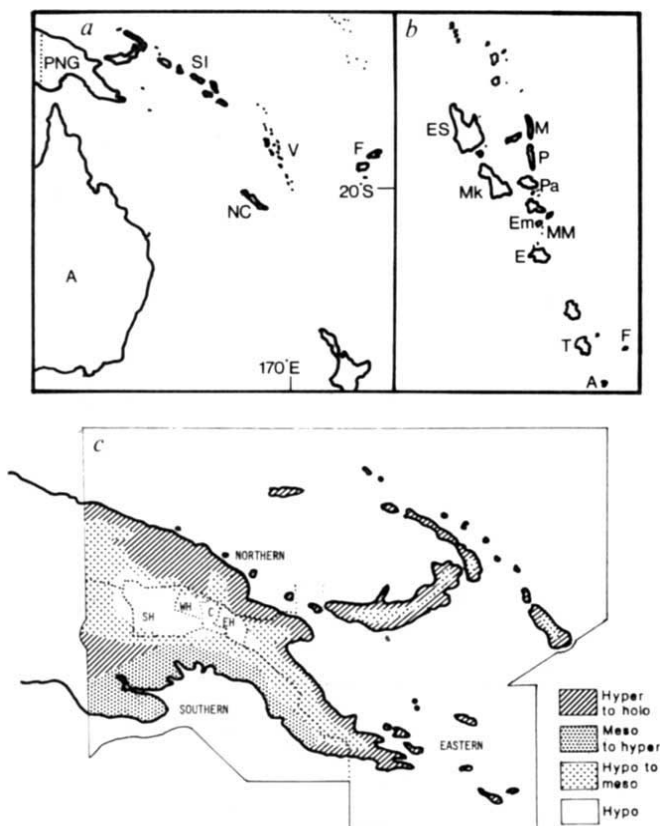


Fig. 1 *a*, Map of the South-West Pacific. Malaria is found west of 170° E and north of 20° S (refs 11, 26). A, Australia; PNG, Papua New Guinea; SI, Solomon Islands; V, Vanuatu; NC, New Caledonia; F, Fiji. *b*, Map of Vanuatu. ES, Espiritu Santo; M, Maewo; P, Pentecost; Mk, Malekula; Pa, Paama; Em, Emae; MM, Makura-Matso; E, Efate; T, Tanna; F, Futuna; A, Aneityum. *c*, Map of Papua New Guinea showing the three coastal regions from which samples were collected (northern southern and eastern), and the four highlands regions (SH, Southern Highlands and Enga Provinces; WH, Western Highlands; C, Chimbu Province; EH, Eastern Highlands) superimposed on estimated malarial endemicity calculated from spleen rate surveys on children aged 2–9 years: holoendemic, >75% splenomegaly; hyperendemic, >50%; mesoendemic, >10%; hypoendemic, <10% (ref. 77). Adapted from refs 15 and 82.

α^+ -thalassaemia gene frequencies correlate strongly with malaria whether the latter varies with latitude or altitude. Moreover, the lack of such correlation for other unlinked polymorphisms and evidence from molecular analysis of α -thalassaemia deletions argue strongly for selection as the mechanism responsible, rather than the accidental consequences of genetic drift or founder effects.

Distribution of α^+ -thalassaemia

Table 1 summarizes the geographical distribution of α -globin genotypes in Melanesia. There are two common deletion forms of α^+ -thalassaemia³²: one involves a loss of 3.7 kilobases (kb) from between the two α -globin genes or removes the $\alpha 1$ gene ($-\alpha^{3.7}$); the other deletion is 4.2 kb long and removes the entire $\alpha 2$ gene ($-\alpha^{4.2}$). Both the $-\alpha^{3.7}$ and $-\alpha^{4.2}$ types were identified wherever the disorder was seen, though the relative proportions of each differed, with the $-\alpha^{4.2}$ predominant only on the north coast of Papua New Guinea. Subtyping³³ of the $-\alpha^{3.7}$ deletions showed that the $-\alpha^{3.7}$ III mutant, which is found only in the South-West Pacific, was much more common than the $-\alpha^{3.7}$ I type³⁴, although the proportions varied with locality. No example of the two- α -gene deletion ($-\alpha/-$) was found in this survey. Non-deletion forms of α^+ -thalassaemia would not have been detected by our screening method; however other evidence

suggests that as in other populations investigated to date, these are much less common in Melanesia than the deletion forms¹³.

It can be seen from Table 1 that α -thalassaemia frequencies generally show a marked cline through Melanesia, falling from almost 70% in north coastal Papua New Guinea in the north-west to less than 10% in New Caledonia in the south-east. Because there is a negative correlation of malaria endemicity with altitude in Papua New Guinea^{12,31}, the most mountainous region that we studied, the data for this region are divided into two groups: highlands and coastal. The highlands samples are subdivided into the five provinces from which they were collected: Enga Province was included in the Southern Highlands, and the Madang highlands samples were from the Bundi region. Coastal samples came from the north (Madang, West and East Sepik Provinces), south (Western, Gulf and Central Provinces) and east (Morobe, Northern and Milne Bay Provinces). No attempt has been made to refine the region of origin further since the objective was to search for gross differences between highlands and lowlands populations. By combining the estimates for different regions (frequencies of 1% for the $-\alpha^{3.7}$ deletion and 3% for the $-\alpha^{4.2}$ deletion), there is an overall figure of 4% for the prevalence of α^+ -thalassaemia in the highlands. Similarly, the respective frequencies for the coastal areas are 12% and 27%, giving a total of 39%. The two key features of these data are thus an altitude-dependent distribution of α -thalassaemia in Papua New Guinea and a latitudinal cline through Melanesia.

Malaria in Melanesia

There have been extensive studies of the occurrence of malaria in the South-West Pacific^{11,15,17–31}. In Papua New Guinea detailed malaria surveys, undertaken since the island was first colonized by Europeans in the late nineteenth century, show that before European contact and the initiation of anti-malaria programmes in the twentieth century, endemic malaria was confined to the coast, the high interior remaining relatively free of the disease^{25–28}. It reaches holoendemic levels along the north coast but is, in general, less prevalent in south coastal provinces; *Plasmodium falciparum* and *Plasmodium vivax* are the predominant parasites (reviewed in refs 12, 15, 24, 31). The first malaria survey in Island Melanesia was performed by Buxton in 1926 in Vanuatu (formerly the New Hebrides)^{17,18}. Further early surveys by French^{20,21} and American^{22,23} workers before and during World War II, respectively, were extended by Black in the 1950s (refs 11, 25–27). Malaria in Vanuatu was found to be seasonal and both *P. falciparum* and *P. vivax* were common but *Plasmodium malariae* was rare. Although throughout the archipelago there is substantial local variation due to differences in the amount of surface water, there was¹⁷ and still is more intense malaria transmission in the northern islands than in the south. The only island east of 170° E in Vanuatu—Futuna—is the only island free of malaria³⁵. Although the most southerly island in Vanuatu—Aneityum—now has malaria¹⁶, historically it too may have been free of the disease²⁰ or have harboured only *P. malariae*³⁶. New Caledonia, the most southerly island in Melanesia, and Fiji, like all of Polynesia to the east, have always been malaria-free^{11,24}. So, in general, as one moves south and east from north coastal Papua New Guinea, the endemicity of malaria declines until it is absent in New Caledonia and Fiji.

α -Thalassaemia and malaria correlation

The results from gene analyses presented here, in conjunction with the findings of surveys of newborn infants for Hb Bart's³⁷, and two small-scale gene mapping investigations of the north coast^{38,39}, indicate a widespread distribution and a consistently high incidence of α -thalassaemia among different coastal groups in Papua New Guinea, and a consistently low incidence above 1,500 m. Detailed reports of malaria occurrence over many generations, such as those available to Siniscalco *et al.*⁴⁰ which demonstrated a correlation between β -thalassaemia and malaria

Table 1 Incidence of α -thalassaemia in Melanesia

	No.	$\alpha\alpha/\alpha\alpha$	$-\alpha/\alpha\alpha$	$-\alpha/-\alpha$	% $-\alpha^{3,7}$	% $-\alpha^{4,2}$	Total % $-\alpha$
Highlands, Papua New Guinea							
Madang (Bundi)	82	75	6	1	0	5	5
Chimbu	89	85	4	0	0	2	2
Eastern Highlands	157	143	14	0	2	3	5
Southern Highlands	190	179	11	0	1	2	3
Western Highlands	21	20	1	0	0	2	2
Total	539	502	36	1	1	3	4
Coastal, Papua New Guinea							
Northern	63	8	24	31	8	60	68
Southern	105	68	28	9	13	9	22
Eastern	20	8	9	3	18	20	38
Total	188	84	61	43	12	27	39
North Solomons	30	9	15	6	23	22	45
Vanuatu							
Espiritu Santo	178	72	75	31	28	10	38
Maewo	169	80	67	22	28	5	33
Pentecost	149	80	56	13	26	1	27
Malekula	21	9	10	2	19	14	33
Paama	25	15	9	1	16	6	22
Emae	85	46	33	6	10	16	26
Makura-Matato	28	23	5	0	3	6	9
Efate	34	20	11	3	17	8	25
Tanna	224	149	69	6	11	7	18
Futuna	56	51	4	1	3	3	6
Aneityum	66	56	10	0	7	1	8
New Caledonia	84	74	10	0	6	1	6

The table shows the number of individuals with different α -globin genotypes and the percentage gene frequencies of α -globin gene deletions in Melanesia. The total number of individuals studied is given in column 1; the number of normals, heterozygotes and homozygotes for single α -globin deletions is given in columns 2 to 4, respectively. The $\alpha\alpha$ genotype^{73,75}, present at a gene frequency of 1%, has been included in the table as $\alpha\alpha$. The percentages of chromosomes with $-\alpha$ deletions are shown in columns 5–7. The Papua New Guinea data are divided according to altitude: coastal samples are from below 1,500 m and highland samples are from above 1,500 m. The Papua New Guinea samples were collected in the larger towns from schools and hospitals attended by villagers from across the island. Both maternal and paternal village of origin were noted and every effort made to sample as widely as possible. The North Solomons samples were collected in Kieta, Bougainville. The islands studied in Vanuatu are listed from north-west to south-east (see Fig. 1). The samples from Vanuatu were both adult and cord blood samples from islands throughout the archipelago, collected between 1980 and 1984. The New Caledonian samples were collected from healthy Melanesians on that island. Extraction of DNA and determination of genotypes by the Southern blot procedure were performed as described previously¹⁰ using 2.85-kb *EcoRI*/*Bam*HI ζ - and 1.5-kb *Pst*I α -specific probes³⁴.

in Sardinia, do not exist in Papua New Guinea, but it is reasonable to assume that malaria has existed for a long time on the island^{29,41}; certainly its occurrence predates European arrival. Although the geography of the island vitiates any simple correlation between altitude, temperature and malarial endemicity (the establishment of a cline for example), our general conclusion, that α -thalassaemia is rare above 1,500 m, agrees with the 1,800–2,000-m upper limit of malaria transmission that Peters and Christian report in their summary of highlands surveys up to 1958 (ref. 42). Moreover, the difference in α -thalassaemia frequency between the south and north coasts correlates with a difference in malarial endemicity^{15,31}.

Table 2 shows that there is a strong association between the earliest recorded endemicities of malaria and the frequencies of α -thalassaemia in different parts of Island Melanesia. As one moves south-east from north coastal Papua New Guinea, through the Solomon Islands and Vanuatu to New Caledonia, the cline in the frequency of α -thalassaemia evident in Table 1 is paralleled by a decrease in malaria endemicity²⁴. New Caledonia, which is free of malaria, has the lowest frequency of α -thalassaemia and in Fiji, which is also free of malaria, α -thalassaemia is rare in Melanesians (D. K. Bowden, unpublished data). Similarly, Futuna, the only island in Vanuatu without malaria³⁵, has the lowest frequency of α -thalassaemia in the group. Early surveys found a higher incidence of malaria in the northern islands of Vanuatu^{18,22,24} and these have a higher incidence of α -thalassaemia than the southern islands. The rank correlation between $-\alpha$ frequencies and malaria endemicity is highly significant (Kendall's $\tau = -0.876$; $P < 0.001$).

Selection hypothesis

It is notable that three different α -gene deletions, $-\alpha^{3,7}$ I, $-\alpha^{3,7}$ III and $-\alpha^{4,2}$, contribute to the high frequency of α -thalassaemia in Melanesia. Analysis of polymorphic restriction enzyme sites in the DNA flanking mutant globin genes allows

some insight into the origin and evolution of these variants. Haplotypes defined by this means have been used to argue for single or multiple origins for particular globin variants⁴³. To investigate the origin of $-\alpha$ deletions in Melanesia, we analysed eight such polymorphisms in the α -gene complex of $-\alpha$ deletion chromosomes⁴⁴. We have reported previously that the predominant $-\alpha^{3,7}$ deletion in this region is of the $\alpha^{3,7}$ III subtype not found in other parts of the world³⁴, and this is associated with a single restriction enzyme haplotype, which argues for a single origin. $-\alpha^{4,2}$ deletions in Melanesia are associated with at least three different haplotypes, none of which is the same as the haplotype associated with six South-East Asian $-\alpha^{4,2}$ mutants (Table 3). Limited analysis of $-\alpha^{3,7}$ I chromosomes

Table 2 Association of α -thalassaemia frequency with malaria endemicity in Melanesia

Island	% $-\alpha$	Malaria endemicity
North coastal PNG	68	Holo to hyper ¹⁵
South coastal PNG	24	Hyper to Meso ¹⁵
North Solomons	45	Hyper ¹⁵
Espiritu Santo	38	Hyper ^{22,25}
Maewo	33	Hyper ¹⁹
Pentecost	27	Hyper ¹⁸
Malekula	33	Hyper ²⁵
Efate	25	Meso to hypo ^{21,25}
Tanna	18	Hypo ⁷⁶
Futuna	6	Absent ³⁵
Aneityum	8	? Absent ^{20,36}
New Caledonia	6	Absent ²⁴

The percentage $-\alpha$ gene frequencies are taken from Table 1, with the islands listed from north-west to south-east (see Fig. 1). Samples (listed in Table 1) from both southern and eastern regions of Papua New Guinea (PNG) are here included in the south coastal group. Extent of malaria endemicity (defined as in ref. 77) is taken from the earliest available surveys, excluding areas where malaria control had been initiated. No early data are available for Emae, Makura-Matato or Paama.

(data not shown) showed that at least two different haplotypes are associated with this variant. Hence, subtyping and haplotype analysis of these chromosomes indicate that the three types of α -thalassaemia deletion have had at least six independent origins. However, of particular interest is the observation that these deletion chromosomes have the same haplotypes as the common normal ($\alpha\alpha$) haplotypes in Melanesia (Table 3). These Melanesian $\alpha\alpha$ haplotypes are unusual in being relatively uncommon in other populations⁴⁴. This suggests that the $-\alpha$ deletion chromosomes now prevalent in Melanesia arose locally on the $\alpha\alpha$ haplotypes common in this region, and were not imported from other malarious areas. The implication of these findings is that some local mechanism is responsible for their elevation to the present high frequencies. As shown in Tables 1 and 3, different types of deletions predominate in different Melanesian populations suggesting, for example, that the $-\alpha^{4.2}$ deletion of haplotype $--+MZ-\text{del}$ may have originated in north coastal Papua New Guinea, and the $\alpha^{3.7}\text{III}$ deletion in northern Vanuatu.

If malarial selection is solely responsible for the high frequencies of α -thalassaemia, it follows that genetic variation at loci unlinked to the α -globin cluster is unlikely to show the same correlation with malaria endemicity.

There exist a large amount of data on blood groups and protein polymorphisms for the inhabitants of Papua New Guinea⁴⁵, and no non-globin locus so far examined has the degree of divergence between highland and coastal populations discovered for α^+ -thalassaemia. To confirm that previous observations apply to our samples, we measured allele frequencies for a number of polymorphic DNA loci, unlinked to the α genes. To maximize the detectable heterogeneity, two types of polymorphisms were examined: those due to changes that alter a restriction enzyme cleavage site and are thus dimorphic, and those due to variation in the copy number of tandem repeats of a consensus sequence (hypervariable regions, HVRs). Whereas the heterozygosity of site polymorphisms can never exceed 50%, the HVRs are multiallelic; they are therefore potentially far more informative than site polymorphisms, and allow finer resolution of genetic relationships between populations. Combining linked polymorphisms into a haplotype also increases the heterozygosity obtainable at a specific locus, and eight polymorphic sites in the β -globin gene cluster^{43,46} were used in this study in addition to site polymorphisms and HVRs. Two probes of known chromosomal location but unknown function were used to look at site polymorphisms. Neither of these probes is linked to the α -globin cluster: probe 2.30 lies on chromosome 2 (ref. 47) and probe 3.6 on chromosome 4 (ref. 48). We examined a further site polymorphism on chromosome 19 with a fragment of the human third complement gene, C3 (ref. 49). The two hypervariable regions examined are on chromosome 11, one 400 base pairs 5' to the insulin gene⁵⁰ and another 1.5 kb 3' to the Harvey *ras-1* oncogene⁵¹; they are arranged in the order β -globin-insulin-Ha-*ras*, with an estimated recombination of 11.6% between β -globin and insulin, and 3.2% between Ha-*ras* and insulin⁵². Unless these samples are from closely related individuals, there is no reason to expect any redundancy of information from looking at all three loci, and indeed no linkage was observed.

There have been relatively few population surveys in Island Melanesia, so there are few protein data to draw on. We therefore analysed the frequencies of two genetic polymorphisms readily detectable by DNA analysis in the same samples from Island Melanesia as used for the α -thalassaemia study. These are the haptoglobin polymorphism, with alleles *Hp*¹ and *Hp*² (ref. 53), and the frequencies of the variant γ -globin genotypes: $-\gamma$ and $\gamma\gamma$ (refs 54, 55). Studies of the phenotypes of these γ -gene variants have shown that they are unlikely to be subject to selection⁴⁶. Tables 4 and 5 summarize the distribution of DNA polymorphisms in Melanesia. A partition of genetic variation⁵⁶ showed a highly significant difference between highland and

Table 3 Haplotypes of $-\alpha$ and $\alpha\alpha$ chromosomes

Population		No.	Haplotype								
			X	S	B	H	Z	A	R	P	
Vanuatu	$-\alpha^{3.7}\text{III}$	19	-	-	+	M	Z	-	-	del	
	$-\alpha^{4.2}$	2	-	-	+	M	Z	-	del	-	
	$-\alpha^{4.2}$	2	+	-	-	S	PZ	+	del	+	
	$\alpha\alpha$	13	-	-	+	M	Z	-	-	-	
	$\alpha\alpha$	4	+	-	+	M	Z	-	-	-	
	$\alpha\alpha$	16	+	-	-	S	PZ	+	-	+	
	$\alpha\alpha$	3	+	-	-	S	PZ	+	-	-	
	$\alpha\alpha$	1	-	-	-	S	PZ	+	-	+	
	$\alpha\alpha$	1	+	+	-	M	PZ	+	+	-	
Papua New Guinea	$-\alpha^{3.7}\text{III}$	2	-	-	+	M	Z	-	-	del	
	$-\alpha^{4.2}$	29	-	-	+	M	Z	-	del	-	
	$-\alpha^{4.2}$	7	-	-	+	M	Z	-	del	+	
	$\alpha\alpha$	14	-	-	+	M	Z	-	-	-	
	$\alpha\alpha$	4	+	-	+	M	Z	-	-	-	
	$\alpha\alpha$	2	+	-	-	S	PZ	+	-	+	
	$\alpha\alpha$	2	+	-	-	S	PZ	+	-	-	
	$\alpha\alpha$	1	-	-	-	S	PZ	+	-	+	
	$\alpha\alpha$	1	-	-	-	S	PZ	+	-	-	
South-East Asia	$-\alpha^{4.2}$	6	+	+	-	M	PZ	+	del	-	

Restriction enzyme haplotype analysis of $-\alpha$ and $\alpha\alpha$ chromosomes from Melanesia. For comparison, data on six deletions from South-East Asians are included. The eight polymorphic sites in the α complex were detected as described in ref. 44. The *Pst*I site 3' to the $\alpha 1$ gene⁴⁴ is not included in these haplotypes. del indicates that the single α -gene deletion removes that polymorphic site, + indicates the presence of a restriction site, - its absence. M and S indicate medium- and small-length alleles, respectively, of the inter-zeta hypervariable region. PZ and Z refer to a $\psi\zeta 1$ and $\zeta 1$ gene, respectively, detected as described previously⁷⁸. X, *Xba*I; S, *Sac*I; B, *Bgl*II; H, inter-zeta hypervariable region; Z, zeta-pseudozeta polymorphism; A, *Acc*I; R, *Rsa*I; P, *Pst*I. Additional probes used to determine these haplotypes were a 0.6-kb *Bam*HI/*Eco*RI fragment from pJW5, a 1.8-kb *Sac*I fragment from pBR ζ , a 1.1-kb *Alu* fragment from pSG21 and a 0.8-kb *Bam*HI fragment from pDH 12 (ref. 44).

coastal regions of Papua New Guinea for $-\alpha$ gene frequencies ($F_{1,6} = 22.04$; $P < 0.001$), but not for other loci. There was no significant association between the frequencies of γ genotypes or haptoglobin alleles and malaria endemicity. Clearly, no allele exhibits an altitude- or latitude-dependent variation comparable to that found for α -thalassaemia.

Genetic drift and founder effect

The most plausible explanation for the observed geographical distribution of α -thalassaemia is that malarial selection is responsible. Support for the malaria hypothesis thus comes from four lines of epidemiological evidence: (1) α -thalassaemia correlates with malaria across two independent variables, namely latitude and altitude; (2) other polymorphic loci unlinked to the α -globin cluster fail to show equivalent variation; (3) a number of phenotypically similar α -thalassaemia variants are present at high frequencies; (4) these mutations arose within the Melanesian populations and have been amplified to high frequencies, implicating a mechanism operating within Melanesia rather than the large-scale import of the variants from elsewhere.

What alternative explanations might accommodate these observations? Genetic drift and founder effects are undoubtedly important in influencing gene frequency variations in small isolated communities⁵⁷⁻⁶⁰. How likely is it that they are responsible for the observed distribution of α -thalassaemia described here?

Examples of the clustering of particular genetic variants on single islands in Vanuatu¹³ suggest that, despite their proximity, historically these islands have remained relatively isolated, increasing the potential for genetic drift. Migration of two populations with different genetic compositions into a single area can readily produce a gene frequency cline. For example, had Melanesia been populated from the south-east by a population with little α -thalassaemia and from the north-west by people in whom it was common, a frequency distribution somewhat similar to that observed might have been generated.

Table 4 Distribution of DNA variants in Melanesia

a Probe	Papua New Guinea highlands						Papua New Guinea coast						
	Madang (Bundi)	Chimbu	Eastern Highlands	Southern Highlands	Western Highlands	Mean gene frequency	Northern region	Southern region	Eastern region	Mean gene frequency			
C3	43 (56)*	42 (78)	53 (18)	58 (83)	50 (10)	48 (245)	48 (33)	52 (62)	50 (8)	51 (103)			
3.6	10 (80)	21 (82)	10 (111)	5 (148)	0 (13)	10 (434)	9 (53)	7 (97)	11 (14)	8 (164)			
2.3	24 (55)	28 (38)	46 (37)	22 (154)	42 (13)	27 (295)	19 (48)	26 (89)	5 (10)	22 (147)			
Insulin HVR class													
1	1	2	4	4	0	3	3	0	0	2			
2	0	0	0	0	0	0	5	4	0	4			
3	0	1	0	4	0	1	0	0	0	0			
4	99 (57)	97 (42)	96 (52)	92 (79)	100 (18)	96 (248)	92 (48)	96 (26)	100 (7)	94 (81)			
Ha-ras-1 HVR class													
1	21	7	7	21	8	14	24	12		18			
2	29	31	26	16	17	23	26	29		28			
3	5	2	0	0	0	1	1	1		1			
4	0	5	6	8	8	6	7	4		5			
5	45	55	61	55	50	55	35	48		42			
6	0	0	0	0	0	0	3	3		3			
Variants	0 (19)	0 (21)	0 (52)	0 (50)	17 (6)	1 (148)	4 (40)	3 (38)		3 (78)			
b	North coastal PNG	South coastal PNG	North Solomons	Espiritu Santo	Maewo	Pentecost	Emae	Efate	Tanna	Futuna	Aneityum	New Caledonia	
	% - γ	5	1	3	11	10	2	12	3	2	13	10	2
	% $\gamma\gamma\gamma$	1	1	2	3	9	4	2	3	1	0	1	8
	% Hp^1	64	71	62	73	76	68	63	61	71	69	61	59
	% - α	68	22	45	38	33	27	26	25	18	6	8	6

a, Percentage gene frequencies of the presence of site polymorphisms as detected by three unlinked probes, and percentages of allele classes as detected by two hypervariable region probes in samples from Papua New Guinea. Two *Bgl*II polymorphisms were examined using probes from a bank of clones used in genetic linkage studies; their chromosomal location is known but not the function of the genomic DNA they contain; they are therefore referred to by laboratory designations: probe 2.30 lies on chromosome 2, probe 3.6 on chromosome 4 (refs 47, 48). An *Sst*I polymorphism detected by a fragment of the third complement component gene (C3) on chromosome 19 (ref. 49) was also surveyed. The insulin gene in phins214 (ref. 79) on a *Rsa*I digest detected four HVR allele classes. Where two alleles appear on a Southern blot to be the same size, it cannot be assumed that they are the same allele, because the technique cannot resolve differences of one or two repeats in a HVR, and also because alleles that are equal in size can have internal sequence differences⁸⁰. Therefore, we refer here to allele classes rather than discrete allele sizes: class 1, 4.7 kb; class 2, 3.9 kb; class 3, 2.6 kb; class 4, 2.1–2.4 kb. Microheterogeneity was particularly obvious with the smaller (class 4) insulin alleles. A *Bam*HI–*Sst*I fragment from the plasmid pBL116-4 containing the Ha-ras-1 HVR²¹ resolved six allele classes in a *Hinf*I digest: class 1, 4.3 kb; class 2, 3.8 kb; class 3, 3.0 kb; class 4, 2.8 kb; class 5, 2.7 kb; class 6, 2.6 kb. Seven alleles could not be fitted into these categories: three (two from the coast and one from the highlands of Papua New Guinea) were of equal size, but the remaining four could not be reconciled either with this size or with each other; they are therefore grouped separately as variants. b, To determine the γ -globin^{54,55} and haptoglobin^{53,81} genotypes in Island Melanesians, the nitrocellulose filters with *Bgl*II-digested DNA samples, prepared previously for determination of α genotypes, were rehybridized, in turn, with a 3.2-kb γ -gene probe⁴⁶ and a 1.5-kb *Pst*I fragment from *Hp*9 (ref. 81). Sample sizes for each island were comparable to those in Table 1 and at least 60 chromosomes were analysed in each case. Some of these haptoglobin, $-\gamma$ and $\gamma\gamma\gamma$ frequencies are reported elsewhere^{46,81}. The $-\alpha$ frequencies from Table 1 are included for comparison.

*Numbers of individuals are shown in parentheses.

In Papua New Guinea there are two ways in which α -thalassaemia gene frequencies might increase by genetic drift or founder effects. First, as part of a substantial population movement, α -thalassaemia could have infiltrated the coastal area on a large scale. Second, an initially homogeneous population with a low prevalence of α -thalassaemia might have become divided genetically as drift elevated the frequency of thalassaemia on the coast and depressed it in the highlands, or the division could have occurred because of colonization of the highlands by a subset of the original settlers lacking the mutation. We believe our data favour selection when viewed from any of these standpoints.

The gene frequencies of the $-\alpha$ deletion, particularly in the north of Papua New Guinea, seem too high to attribute to random fluctuations unless the founding population was extremely small. Furthermore, genetic drift should produce uneven variation from island to island, as seen for the $-\gamma$ and $\gamma\gamma\gamma$ frequencies, rather than a frequency cline. Moreover, assuming that the $-\alpha$ chromosome in most of the founding populations of these islands was less common than the normal $\alpha\alpha$ chromosome, in the absence of selection, the deletion chromosome should have been lost completely on some islands through genetic drift, as appears to have happened with the $-\alpha^{3,7}$ III mutation when it was carried out into the central Pacific³⁴, where there is no malaria²⁴, by the settlers of Polynesia.

That this has not occurred in the malarial regions of Melanesia suggests that selection is involved.

With the exception of some recent back-migration from Polynesia, all known migrations into Melanesia have come from the north-west⁶¹. The 'outlier' islands of Futuna and Emae in Vanuatu have ethnically Polynesian populations and, culturally, there is probably more Polynesian influence in the south than in the north of Vanuatu⁶¹. Similarly, a relatively recent admixture of Polynesian genes into the south coastal population of Papua New Guinea may, in part, explain the lower overall prevalence of α -thalassaemia there than on the north coast. However, such back-migration has almost certainly been too limited to explain more than a minor part of the observed α -thalassaemia cline. For example, the 'Polynesian' population of Emae has a much higher α -thalassaemia frequency than anywhere in Polynesia⁶². Similarly, although a late migration from the north-west by a very large population with a high frequency of α^+ -thalassaemia could have produced a $-\alpha$ gene frequency cline, this is inconsistent with the haplotype data which indicate that most of the $-\alpha$ chromosomes found in Melanesia had local origins and have not been imported from South-East Asia. Furthermore, no simple migration model could explain the generation of a cline made up of different proportions of the various molecular types of $-\alpha$ deletions in different locations.

Similar arguments apply within Papua New Guinea. Here

Table 5 Distribution of β -globin haplotypes in Papua New Guinea highland and coastal populations

Highlands	No.	5' Haplotype									
		+- - - -	- + - + +	- + + - +	- + - + -	- - - + -	- - - + +	- + - + +	- + + + +	- - - - -	- - + - -
Southern Highlands	19	69	18	5	5	3	0	0	0	0	0
Eastern Highlands	20	59	7	5	10	3	10	0	3	3	0
Total	39	64	13	5	8	3	5	0	1	1	0
Coast	No.	3' Haplotype									
		++ -	+ + +	+ - -	- - +	+ - +	+ + +				
Madang	19	55	16	0	0	0	16	5	5	0	3
Southern Highlands	19	53	18	8	13	5	3				
Eastern Highlands	18	56	19	0	6	11	8				
Total	37	54	19	4	10	8	5				
Coast											
Madang	16	47	22	0	19	9	3				

Percentage frequencies of β -globin gene complex haplotypes in two highland provinces and the north coast of Papua New Guinea. The β -globin haplotype used here consists of five sites reported previously and referred to as 5' haplotype, and three sites (*Ava*II, *Hind*III and *Bam*HI) combined into a 3' haplotype⁴³; 'v' indicates the presence of an additional *Hind*III site 11 kb 3' to the β -globin gene⁴⁶.

again three subtypes of α^+ -thalassaemia are found at elevated frequencies. Genetic drift or disturbance caused by new population influx should have affected other gene frequencies as well as α -thalassaemia. Unless by chance we, and others, have looked only at unaffected loci, drift acting to divide the population can be excluded as an explanation of the observed distribution of α -thalassaemia. Similarly, immigration leaving existing gene frequencies unaltered would have been possible only if the invaders were genetically similar to the resident Papuans except at the α -globin locus. While this possibility cannot be formally excluded, it runs counter to evidence from other disciplines.

Archaeological and linguistic data indicate a definite but small Austronesian admixture to the Papuan stock^{63,64}, and this is supported by genetic studies^{65,66}. There exist a large amount of data on blood group and serum protein polymorphisms in Papua New Guinea⁴⁵. Markers that distinguish between highland and coastal populations are either common alleles which show minor but significant variation between populations (for example, ABO and MNS blood groups⁶⁷) or alleles occurring at low frequencies in one or other of the populations (MDH⁴, ref. 68; or Ge^{a+}, ref. 69). This argues that immigration into coastal areas produced only minor changes in gene frequencies. Hence if α -thalassaemia had been imported it would have resulted in only low frequencies of the condition in the total coastal population.

The most plausible explanation for the observed difference is, therefore, that selection has acted on mutations arising within the original settlers of Melanesia.

Discussion

This microepidemiological study in Melanesia strongly implicates malaria as the selective agent responsible for the high prevalences of α -thalassaemia in certain tropical and subtropical regions. Limited data documenting the absence of α -thalassaemia in populations historically free of malaria support this conclusion⁷⁰. The one apparent exception is the presence of α -thalassaemia at gene frequencies of ~7% in parts of Polynesia⁶², all of which is free of malaria²⁴. However, detailed molecular analysis of the $-\alpha$ deletion found in Polynesia shows that it is identical to the $-\alpha^{3,7}$ III deletion prevalent in Island Melanesia. It seems probable, therefore, that α -thalassaemia was carried out into Polynesia by the original migrants from malarious Melanesia, its present patchy distribution reflecting the influence of genetic drift and founder effects over the past 3,000 years^{34,62}.

The mechanism whereby α -thalassaemia might confer protection against *P. falciparum* malaria is unclear. *In vitro* culture studies have not demonstrated any abnormality of invasion or growth of the parasites in the red cells of α - or β -thalassaemia heterozygotes, except in cases where the cells were maintained under conditions of unusual oxidant stress⁷¹. Furthermore, with the exception of the newborn period when the red cells contain increased levels of Hb Bart's, the haematological abnormalities

in the α -thalassaemia carrier states are extremely mild and there are no changes in haemoglobin constitution. Thus the protective effect of α -thalassaemia against malarial infection must be extremely subtle and may not constitute a direct deleterious effect on parasite development, such as has been shown in the case of the red cells of sickle cell carriers⁷¹.

Although the mechanism involved remains obscure, the epidemiological data do provide some indication of the magnitude of the apparent selective advantage conferred by α^+ -thalassaemia with respect to malaria. It is unclear whether the observed gene frequencies in Melanesia are at equilibrium or whether the single α -gene deletion is still increasing in frequency. If the latter is true, because Island Melanesia has been inhabited for over 3,500 years and Papua New Guinea for much longer⁶¹, the observation that the $-\alpha$ chromosomes have not yet reached their equilibrium frequency would suggest that the selective advantage is relatively small. On the other hand, if the present frequencies are at or near equilibrium, the selective advantage in the presence of malaria must be balanced by some disadvantage associated with homozygosity for α^+ -thalassaemia. The mildness of the latter condition, which contrasts markedly with homozygosity for β -thalassaemia or Hb S, would again suggest that the balancing selective advantage of α^+ -thalassaemia against malaria is small. This may explain the failure to detect differences between the $-\alpha$ and $\alpha\alpha$ haplotypes in *in vitro* invasion or growth assays using *P. falciparum* in culture^{71,72}. It seems that these data on α -thalassaemia in Melanesia provide a remarkable example of how a small selective advantage can lead to large variations in gene frequencies between human populations.

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LETTERS TO NATURE

PKS1345+125: a Seyfert nucleus merging with a powerful radio galaxy

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The bright compact radio source PKS1345+125 (4C 12.50) has been optically identified with a magnitude 17 galaxy with a redshift $z=0.122$ (refs 1, 2) and an unusual spectrum³. Here we show it to be an elliptical galaxy with a compact radio source coincident with its optically undistinguished nucleus. An unresolved radio-quiet Seyfert nucleus with the same redshift as the elliptical galaxy is offset by 1.8 arc s from the radio source. The Seyfert nucleus is remarkable for its location in an elliptical galaxy, for its extreme far-infrared flux, and for the very low density in the broad-line region. We present a model of 1345+125 involving the dynamical merger of an elliptical galaxy with a spiral. The merger has stripped the spiral galaxy of its interstellar gas, fuelling the nuclear engines in both galaxies, and allowing the broad-line region to stream out freely. The elliptical has acquired an envelope, and is becoming a radio-loud cD galaxy.

PKS1345+125 is a suspected low-frequency radio variable, showing fractional flux variations of 30% on timescales of 4.5 yr (refs 4, 5) at a frequency of 408 MHz. If these variations are intrinsic to an incoherent synchrotron-emitting source, then relativistic effects are affecting significantly the observed properties of this source⁶. The radio source is in the VLA (Very Large

Table 1 Photometric data for PKS 1345+125

Waveband	Wavelength (μm)	log flux (Jy)	log frequency
J*	1.20	-2.61 ± 0.04	14.40
H*	1.64	-2.48 ± 0.02	14.26
K*	2.19	-2.46 ± 0.02	14.14
L*	3.80	-2.08 ± 0.05	13.90
1†	12	-0.6	13.40
2†	25	-0.17	13.08
3†	60	0.30	12.70
4†	100	0.31	12.48

* 7.5-arc s aperture, 16-arc s chop north-south.

† IRAS point-course catalogue⁸.

Array) calibration list, so that reliable structural and positional data are available (ref. 7 and J. J. Condon, personal communication). More than 95% of its flux is contained in a region smaller than 0.5, 0.15 and 0.05 arc s at 20, 6 and 2 cm respectively (J. J. Condon, personal communication).

In a study of the optical spectrum, Grandi³ noted the very broad forbidden lines, but classified the spectrum as that of a narrow-line radio galaxy because of its excitation level. The order-of-magnitude discrepancy between the velocity width in this object and in other narrow-line radio galaxies was noted, but did not overrule the classification. The absorption-line redshift³ due to the underlying galaxy is consistent with that of the emission-line source.

The optical identification was classified as an S0 galaxy¹, presumably because the image is noticeably irregular on sky survey plates. To study the morphology in more detail, we

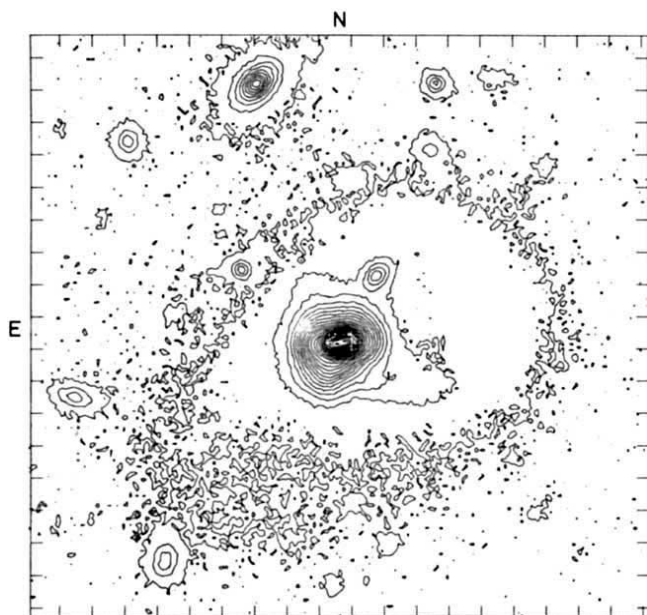


Fig. 1 R-band CCD image of PKS1345+125. North is at the top and East is to the left. The tick marks on the axes occur every 10 pixels, or 4.9 arc s. The eastern nucleus of the dominant galaxy is coincident with the radio source; the western nucleus is the Seyfert source.

obtained B- and R-band CCD (charge-coupled device) frames with an RCA CCD on the Anglo-Australian telescope in April 1985. Several exposures were obtained in each colour with the telescope offset between exposures to remove the effects of bad pixels and cosmic-ray events. All frames in each colour were bias-subtracted, defringed, flat-fielded, offset to a common centre and summed. Part of the resulting R frame is shown in Fig. 1, and shows the galaxy to be a very distorted, double-nucleus, giant elliptical in the centre of a cluster. The important features of this galaxy are its two nuclei, its distorted isophotal structure and its extensive asymmetric envelope.

The outer galactic isophotes in Fig. 1 correspond to a projected linear diameter of $110 h^{-1}$ kpc, where h is the Hubble constant in units of $100 \text{ km s}^{-1} \text{ Mpc}^{-1}$. This galaxy then has the physical dimensions of a cD galaxy, consistent with its location at the centre of a cluster. The distorted isophotal structure indicates a recent (on a dynamical timescale) interaction in which the perturbations to the local gravitational potential are comparable to the potential in the outer parts of the galaxy. The luminosity profile is not consistent with an $r^{1/4}$ law.

Coordinates for the two nuclei have been determined relative to the nine compact images on the CCD image which are also visible on a UK Schmidt telescope plate, which in turn were measured relative to a set of AGK3 stars. The resulting positions (RA, dec. (1950.0)) for the two nuclei are: eastern nucleus, (13 h 45 min 06.20 s $\pm$ 0.02 s, +12° 32' 20.06" $\pm$ 0.30"); western nucleus, (13 h 45 min 06.09 s $\pm$ 0.02 s, +12° 32' 20.57" $\pm$ 0.30"). The VLA position (ref. 7 and J. J. Condon, personal communication) of the compact radio source is (13 h 45 min 06.17 s $\pm$ 0.01 s, +12° 32' 20.3" $\pm$ 0.1"). The eastern nucleus is most closely coincident with the centre of the dominant galaxy, is coincident with the compact radio source and is very red. The bluer western nucleus is unresolved in our CCD image, as it was during spectroscopic observations at La Palma, indicating a size of <0.9 arc s.

PKS 1345+125 was detected as a point source by IRAS (the Infrared Astronomy Satellite) at 100, 65, and 25 μm . Only an upper limit to the flux is available at 12 μm (ref. 8). If the infrared flux were from the elliptical galaxy, it would be unique in the IRAS survey; an identification of the infrared flux source with

the emission-line source is therefore suggested. Near-infrared photometry was obtained in January 1982 (before the IRAS mission) with the UK Infrared Telescope. This shows substantial excess flux at 3.8 μm relative to that expected from an elliptical galaxy. The observations were obtained through a 7.5-arc s aperture including both nuclei, relative to positions 16-arc s north and south and are detailed in Table 1.

The IRAS data cannot be fitted by a single-temperature black body, with emissivity varying as frequency or (frequency)², which encompass the usual range of assumed parameters. Sufficiently few data points are available for wavelengths longer than 3 μm that it is possible to fit the data with a power law having a break near 50 μm . Such a fit is steeper than usually observed (spectra index = -2.36), and is not required by the data.

The most conservative assumption possible is that a black body with emissivity ϵ proportional to frequency and fitted to the 60- and 100- μm data represents the total infrared flux. In this case the required temperature is $T=47$ K, the resulting infrared flux is $F_{\text{IR}}=1.25 \times 10^{-13} \text{ W m}^{-2}$ and the absolute luminosity is $L_{\text{IR}}=2 \times 10^{45} h^2 \text{ erg s}^{-1}$, or $L_{\text{IR}}=5 \times 10^{11} h^2 L_{\odot}$, with $L_{\odot}$ the solar luminosity. Even with these very conservative assumptions, this source is more luminous than 90% of those in the IRAS catalogue⁸.

The minimum possible radius for the source may be calculated to be $r_{\text{IR}}=220 h^{-1} \epsilon^{-1/2} \text{ pc}$, where ϵ is the emissivity at 60 μm . Assuming that $\epsilon \approx 1$, as representative for dust in our Galaxy, leads to a minimum physical size, $r_{\text{IR}} \approx 220 h^{-1} \text{ pc}$, and a corresponding minimum angular size, $\theta_{\text{IR}} \approx 0.1 \text{ arc s}$. We emphasize that this source is almost certainly coincident with the optical emission-line source.

Optical spectra with a resolution of $\sim 1 \text{ \AA}$ were obtained in July 1982 and February 1984 with the du Pont telescope and reticon array detector at the Las Campanas observatory, and in May 1985 with the Isaac Newton telescope and an IPCS detector at La Palma. The La Palma observations used a 1-arc s slit in excellent seeing (~ 0.9 arc s with both nuclei visible) to check for spatially resolved emission. None was found. There is no optical line emission except from the unresolved radio-quiet western nucleus, and no continuum is seen from the line-emitting source.

The remarkable feature of the optical spectrum is the width, complexity and asymmetry of the [O III] 4,959+5,007 $\text{\AA}$ (Fig. 2a) and H α + [N II] 6,563+6,548+6,583 $\text{\AA}$ (Fig. 2b) emission-line profiles. The half-width at zero intensity of these forbidden lines is $\sim 5000 \text{ km s}^{-1}$. Line fluxes for the important diagnostic lines [O I] 6,300 $\text{\AA}$ and [S II] 6,716+6,731 $\text{\AA}$ are available only from the narrow-slit La Palma observations, with consequent large uncertainties, as seen by a comparison of the line fluxes determined here (Table 2) with the large-aperture data of Grandi³.

The line ratios of Table 2 provide a powerful diagnostic of the ionization mechanism. We can use all five diagnostic

Table 2 Spectral data for the emission-line source

Emission line	Flux* ($10^{-16} \text{ erg cm}^{-2} \text{ s}^{-1}$)
[O II] ($\text{\AA}$) 3,726, 3,729	85
[Ne III] 3,869	25
[S II] 4,068, 4,076	15
[O I] 4,363	2
H β 4,861	20
[O III] 4,959	80
[O III] 5,007	170
[O I] 6,300	10
H α + [N II] 6,563, 6,548, 6,583	130
[S II] 6,716, 6,731	40

* Due to different slit losses in the source and the flux standard, these values are expected to be accurate to 50% at 5,000 $\text{\AA}$, to be systematically too high by $\sim 100\%$ in the blue, and too low by $\sim 100\%$ in the red.

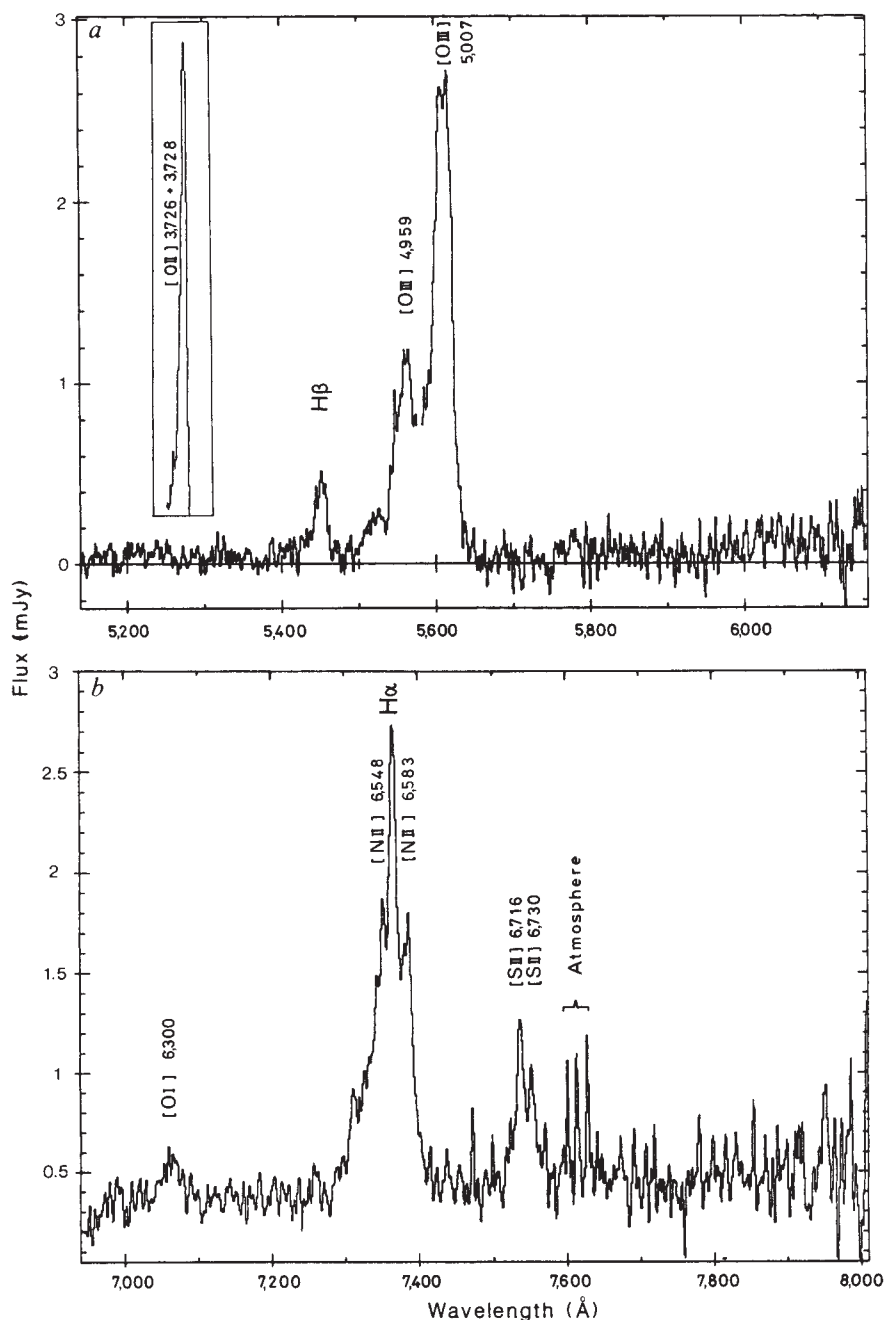


Fig. 2 Two portions of the optical spectrum of the western nucleus in Fig. 1. *a* shows the H β and [O III] 4,959 + 5,007 Å lines; the [O II] 3,726 + 3,728 Å doublet is shown in the inset. The width, complexity and asymmetry of the line profiles is evident. No continuum emission is detected. *b* shows the red diagnostic lines. The narrow emission features near 7,600 Å are atmospheric A-band artefacts, but illustrate the instrumental resolution. The underlying galactic continuum has not been subtracted here.

diagrams discussed by Baldwin *et al.*⁹: two examples are shown in Fig. 3. Clearly, the emission-line spectrum is indistinguishable in line ratios from that of a classical Seyfert galaxy with ionization provided by a central photoionizing source. In this case we can use the various [S II] and [O III] line ratios to derive¹⁰ an electron density $N_e \approx 300 \text{ cm}^{-3}$ and a temperature $T \approx 10^4 \text{ K}$. These values are consistent with our observed line ratios and our null detection of [O III] 4,363 Å. The remarkable feature of the emission-line source is therefore not its velocity field, which is typical of other Seyfert galaxies, but its extremely low electron density. We discuss this further below.

The observed H α luminosity ($\sim 10^{42} \text{ erg s}^{-1}$) allows a calculation of the radius of the emitting volume, $r_{H\beta}$, as a function of the filling factor of line-emitting gas, η , to give $r_{H\beta} \approx 300 \eta^{1/3} \text{ pc}$, and a corresponding upper limit on the source angular size, $\theta_{H\beta} \leq 0.2 \text{ h arc s}$, consistent with our observations.

We now outline a self-consistent model of this object. The extreme far-infrared luminosity (unknown for an elliptical galaxy) and the limited photometric evidence suggest the coin-

cidence of the IRAS and the optical emission-line sources. If they are indeed, then the coincidence of the upper limit on the size of the line-emitting region and the lower limit on the size deduced for the dust source requires that the filling factor for the line-emitting gas is of order unity, the size of the emitting region is $\sim 300 \text{ pc}$, the mass of enclosed H α -emitting gas is $\sim 10^8 M_\odot$ (where $M_\odot$ is the solar mass) and the velocity field has a range of $\sim 10^4 \text{ km s}^{-1}$. For this gas to be gravitationally bound requires a mass of $\sim 10^{12} M_\odot$ within 300 pc. Clearly, the gas must be expanding. If this expansion is driven by radiation pressure from a central accreting black hole, its mass may be calculated from $L_{\text{Edd}} \approx 10^{46.2} (M/10^8 M_\odot) \text{ erg s}^{-1}$, for radiation at the Eddington limit¹¹. Our observed total luminosity of $2 \times 10^{45} \text{ h}^2 \text{ erg s}^{-1}$ then implies a mass of $\sim 10^7 \text{ h}^{-2} M_\odot$.

If the dust and the expanding emission-line gas coexist, the line asymmetry is readily understood¹² as due to obscuration of the far side (redshifted wing) of the gas cloud.

Why then does this source have a much lower density in the broad-line region than do other Seyfert nuclei? The obvious

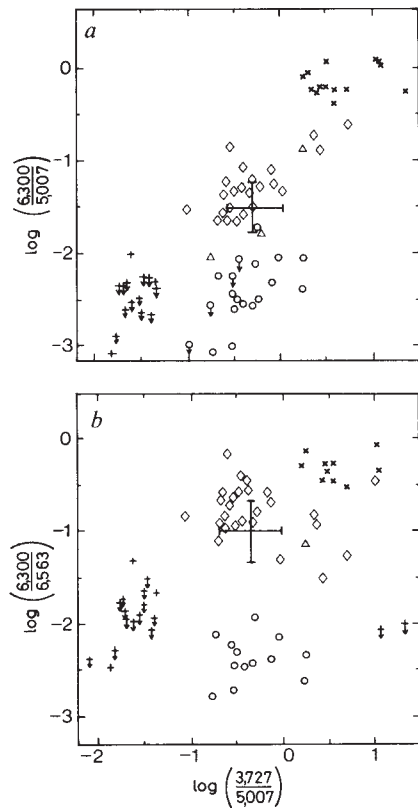


Fig. 3 Diagnostic diagrams showing the relationships between log intensity ratios (see ref. 10): *a*, $\log(6,300 \text{ \AA}/5,007 \text{ \AA})$ versus $\log(3,727 \text{ \AA}/5,007 \text{ \AA})$; *b*, $\log(6,300 \text{ \AA}/6,563 \text{ \AA})$ versus $\log(3,727 \text{ \AA}/5,007 \text{ \AA})$. Plus signs, planetary nebulae; Open circles, normal H II regions; the crosses are thought to be shock-heated galaxies and the diamonds are thought to be photoionized (Seyfert) galaxies. Arrows indicate upper limits. The large cross represents the emission-line source discussed in the text, with the length of the arms indicating a factor of two uncertainty. This source is seen to lie in the Seyfert part of both diagrams.

solution is its location, in transit through the deep potential well of the giant elliptical. Tidal stripping of a spiral galaxy can efficiently remove both the stars and the interstellar medium in such circumstances (this can be estimated from Roche-limit arguments comparing the local potential wells of the two galaxies). There is then no containing medium or potential gradient to resist free expansion of the gas. Similarly, efficient fuelling of the nuclear engine is expected from tidal deformations of the potential well^{13,14}. The lack of optical line emission from the compact radio source is also explicable if the accretion rate into the centre of the elliptical is low¹⁵. This situation will no doubt change dramatically if the Syfert nucleus spirals into the centre of the elliptical.

The disturbed optical isophotes of the elliptical suggest that the interaction has been both violent (perturbations of order unity to the local gravitational potential) and recent. Phase mixing will restore the symmetry of the structure on a timescale D/σ , with D a typical distance and σ a velocity dispersion. For $D \approx 10\text{--}50$ kpc and $\sigma \approx 400$ km s⁻¹, this time is $10^7\text{--}10^8$ yr. As the structure becomes symmetric, the galaxy will acquire an extensive, shallow-profile envelope, indicative of a cD galaxy. This system therefore provides an example of the importance of dynamical evolution to radio galaxies and first-ranked cluster ellipticals.

The radio structure of the compact source could be measured by very-long-baseline interferometry. At this redshift, distances of ~ 1 pc are measurable, allowing a detailed study of a low-

frequency radio variable at high physical resolution. In this case, however, the complexity of the system makes it likely that the apparent variability at low radio frequencies is not intrinsic to the source, but is an effect of propagation through the interstellar medium of the host galaxy. This hypothesis is amenable to direct observational test, as is the coincidence of the optical emission line and the far-infrared sources.

The Isaac Newton telescope is operated on the island of La Palma by the Royal Greenwich Observatory in the Spanish Observatorio del Roque de los Muchachos of the Instituto de Astrofísica de Canarias. G.G. is a visiting associate at the Mt Wilson and Las Campanas Observatories, Carnegie Institution of Washington.

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A compact radio source in the nucleus of M87

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The nucleus and jet of the giant elliptical galaxy M87 have been studied at radio wavelengths with resolutions ranging upwards from a few milliarc seconds^{1–3}. The higher-resolution maps show that the nucleus consists of a bright central region with a feature with smoothly decaying brightness pointing in the direction of the larger-scale radio/optical/X-ray jet. Moreover, these maps indicate that collimation of the jet occurs within 10 mas of the nucleus (that is, within 1 pc of the nucleus, as M87 lies at a distance of 12–20 Mpc⁴). We report here high-resolution, very-long-baseline interferometric (VLBI) observations at 1.35 cm wavelength, which reveal the existence of a compact core (≤ 0.2 mas) and an associated extended feature (~ 0.3 mas), again pointing in the same direction as the large-scale jet. Our observations show that the radio jet is formed on a size scale of $\leq 10^{-2}$ pc, thus strengthening the evidence for the presence of a massive black hole in the nucleus of M87.

Optical observations of the nucleus of M87 (Virgo A, 3C274) show a central luminosity spike of size < 1 arc s and a velocity dispersion which increases towards the centre^{5–8}. These data suggest the presence of a massive compact object of mass $5 \times 10^9 M_\odot$ (ref. 5), where $M_\odot$ is the solar mass. Accretion onto this object may give rise to intense activity, as dramatically demonstrated by the radio¹/optical⁹/X-ray¹⁰ jet extending for ~ 30 arc s to the west of the nucleus. The mechanism of origin of the jet (and of extragalactic jets in general) is unclear¹¹ and its investigation requires the highest available resolution to observe the region where the jet is first produced.

Our observations were made on 8 February 1985 with the VLBI Mk III recording system using 28-MHz bandwidth. The telescopes used were the Max-Planck-Institut für Radioas-

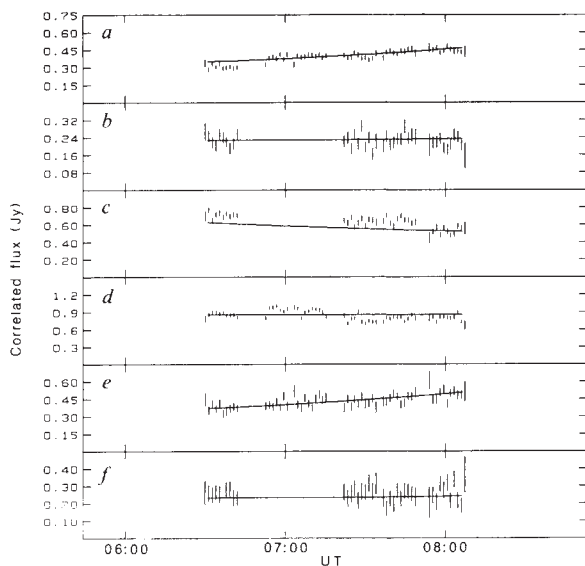


Fig. 1 Plots of correlated flux density on each baseline versus Universal Time on 8 February 1985 for $\lambda = 1.35$ cm observations of M87. *a*, Effelsberg (Eff)–Haystack (Hay); *b*, Eff–Owens Valley (Owe); *c*, Hay–Owe; *d*, Eff–Onsala (Ons); *e*, Hay–Ons; *f*, Owe–Ons. Solid lines, model visibilities fitted to the data.

tronomie (MPIfR) 100 m at Effelsberg, FRG, the Onsala Space Observatory 20 m, Sweden, the Haystack Observatory 36 m and in the United States the Owens Valley Radio Observatory (OVRO) 40 m, giving a maximum baseline of $6 \times 10^8 \lambda$. M87 was observed for 2 hours. Additionally, brief observations of 3C273 and 1611+343 were made; the first as a ‘fringe-finder’ to aid the setting-up of the Mk III analysis package and the latter to calibrate the fringe amplitudes. The data, recorded on magnetic tape, were processed at MPIfR, Bonn, to produce correlation amplitudes and phases (integrated over 16 s). Later, these were corrected for the individual receiver system temperatures and antenna gains and averaged arithmetically to 80 s. There was no evidence for a significant systematic decrease of the corrected fringe amplitude with baseline length for the source 1611+343, thus indicating that this source was suitable as a calibrator. The average flux density of this source was 0.84 ± 0.05 Jy, whereas observations of M87 gave an average flux density on the shortest baseline (Onsala–Effelsberg, $6.1 \times 10^7 \lambda$) of 0.83 ± 0.08 Jy, decreasing with increasing baseline length to 0.23 ± 0.03 Jy for the longest baseline (Effelsberg–OVRO) (see Figs 1, 2). Some idea of the reliability of the calibration can be obtained by noting that, for the calibrator source 1611+343, the closure amplitudes¹² were within 21% of unity and typically were much closer than this ($\sim 8\%$), showing that this source was largely unresolved. The closure amplitudes for M87 were greater than 2, reflecting strong resolution effects.

The angular structure of M87 was obtained by fitting the Fourier transform of a model of the sky brightness to the calibrated data using a least-squares method. The model in best agreement with the observations consisted of an unresolved core (< 0.2 marc s) of flux 0.22 Jy and an extended component 0.33 marc s in diameter of flux 0.65 Jy, offset from the core by 0.06 marc s to the west. Figure 2 shows the fit between this model and the observed amplitudes as a function of projected baseline length. Observed closure phases¹² (Fig. 3) clearly show an offset from 0° , showing that the source is asymmetric and extended to the west. As an independent test of this model, a map of the source was produced using the ‘hybrid mapping’ technique of Readhead and Wilkinson¹². This clearly showed a western extension of the source in spite of the large north–south size of the synthesized beam. Figure 4 shows a contour plot of the map using a restoring beam of diameter 0.15 marc s. This is also a

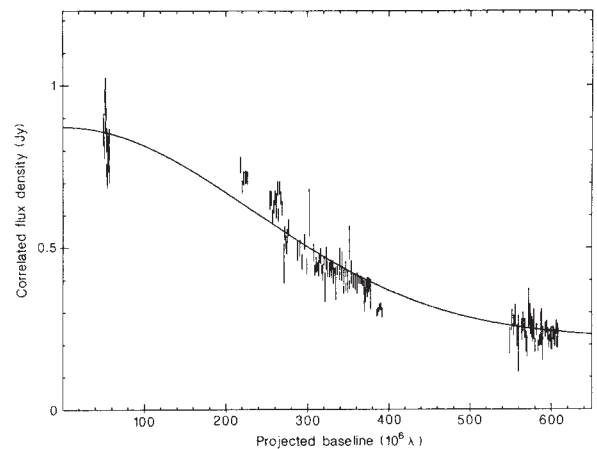


Fig. 2 Plot of the observed amplitudes for M87 against projected baseline length. The solid line superimposed on the data represents the variation of amplitude with projected baseline length for the two-component model derived from model fitting.

representation of the source structure found by model fitting.

The VLBI observations by Reid *et al.*² with a resolution of 8 marc s show that the inner jet lies at a position angle of 288° . The limited hour-angle range of our observations is not sufficient to constrain the position angle of the model to better than about a radian, but the existence of this extension in the same general direction as the larger jet suggests continuity over a size range of more than 5 orders of magnitude, with formation of the jet occurring within < 0.2 marc s ($\approx 10^{-2}$ pc) of the nuclear core.

Blandford and Rees¹³ suggested that jets may be produced by fluid dynamical processes in high-entropy gas confined by a gravitational potential well. Collimation of the jets would occur via ‘nozzles’ situated at roughly one scale height from the centre of the well. The potential well could be produced by a dense star cluster or compact massive object. In the former case, we can follow Jenkins⁸ and use the optically measured velocity dispersion, ΔV , to estimate the mass M contained within a radius r , that is, $M \approx 3r(\Delta V)^2/G$ (where G is the gravitational constant). The observations of Stauffer and Spinrad⁷ indicate a

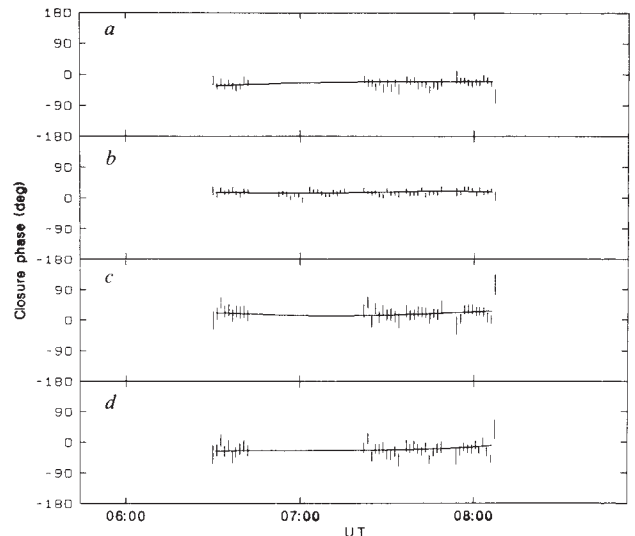


Fig. 3 Plots of closure phase versus Universal Time for 1.35-cm observations of M87. *a*, Eff–Hay–Owe; *b*, Eff–Hay–Ons; *c*, Eff–Owe–Ons; *d*, Hay–Owe–Ons. Solid lines, model closure phases fitted to the data. A symmetric source would give a closure phase of 0° .

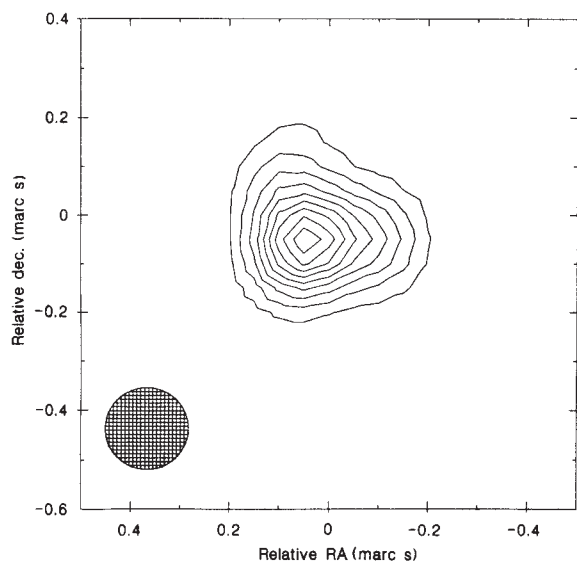


Fig. 4 Contour map of the nucleus of M87 at 1.35 cm wavelength. A circular gaussian restoring beam of diameter 0.15 marc s (shown in the bottom left-hand corner) has been used. Contours are shown at 10, 20, 30, 40, 50, 60, 70, 80 and 90% of the peak brightness of 0.34 Jy per beam.

value of $\sim 400 \text{ km s}^{-1}$ at 1 arc s from the nucleus, but 'seeing' and any departures from a spherically symmetric gravitational system mean that this is a lower bound on ΔV . Thus, if a star cluster has produced the potential well, then it must contain a mass of at least $10^6 M_\odot$ within a radius of 10^{-2} pc . The star density within such a cluster would be extremely high (assuming a reasonable mass function for the cluster), and consequently the cluster would have a relaxation time of $\sim 10^5 \text{ yr}$. This cluster would then evaporate in a time which is brief when compared with the lifetime of the large-scale structure of the source ($\sim 10^8 \text{ yr}$)¹⁴, suggesting that such a model is unlikely.

Accretion disks orbiting massive black holes have been proposed in order to explain many observed features of active galactic nuclei such as that of M87. In particular, the 'funnels' formed in thick accretion disks can collimate the ejected material to form jets¹¹. Such disks may be characterized naturally by Q , the ratio of the outer radius to the inner¹⁵. Thick accretion disks with large values of Q would produce well-collimated jets. If a massive black hole of mass $5 \times 10^9 M_\odot$ exists in M87, then, for a funnel size of $< 10^{-2} \text{ pc}$ as suggested by our observations (assuming that the unresolved core encompasses the entire accretion disk), Q must be less than 30. However, much higher values of Q are required for the production of highly collimated jets, such as that of M87.

The radio power in the jet, estimated from the stored energy in the large-scale radio source¹⁴ divided by the lifetime, is $\sim 10^{36} \text{ W}$. This amount of power is equal to the Eddington luminosity of accretion onto a black hole of mass $\sim 10^6 M_\odot$. Our observations indicate a Q value of $\sim 10^3$ for a disk surrounding a black hole of this mass: such an arrangement would be more able to produce a collimated jet. It has been pointed out¹¹ that if a very massive black hole of mass $5 \times 10^9 M_\odot$ were present in the nucleus of M87 it would have to be extremely underluminous, and, in view of the uncertainties^{16,17} of the existence of such a massive object, a black hole of $10^6 M_\odot$ surrounded by a thick accretion disk fed from material from a much larger star cluster ($> 0.1 \text{ arc s}$) is a more likely model. The resolution given by the Space Telescope should enable further studies of this star cluster to be made. Also, the proposed Quasar VLBI satellite may yield more information on the radio jet and perhaps set tighter limits on the massive object in M87.

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observatories of MPIfR, Owens Valley, Haystack and Onsala for assistance with the observations, and acknowledge the help of the staff of MPIfR, Bonn, in processing the VLBI tapes. VLBI at Owens Valley Radio Observatory and at the Haystack Observatory of the Northeast Radio Observatory Corporation (NEROC) is supported by the NSF. W.J. thanks SERC for a studentship.

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On the depletion of Antarctic ozone

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Recent observations by Farman *et al.*¹ reveal remarkable depletions in the total atmospheric ozone content in Antarctica. The observed total ozone decreased smoothly during the period from about 1975 to the present, but only in the spring season. The observed ozone content at Halley Bay was $\sim 30\%$ lower in the Antarctic spring seasons (October) of 1980-84 than in the springs of 1957-73. No such obvious perturbation is observable in other seasons, or at other than the very highest latitudes in the Southern Hemisphere, and the magnitude of the observed change there far exceeds climatological variability². We present here balloonsonde ozone data^{3,4} which show that these ozone changes are largely confined to the region from about 10 to 20 km, during the period August to October. We show that homogeneous (gas phase) chemistry as presently understood cannot explain these observed depletions. On the other hand, a unique feature of the Antarctic lower stratosphere is its high frequency of polar stratospheric clouds⁵, providing a reaction site for heterogeneous reactions. A heterogeneous reaction between HCl and ClONO₂ is explored as a possible mechanism to explain the ozone observations. This process produces changes in ozone that are consistent with the observations, and its implications for the behaviour of HNO₃ and NO₂ in the Antarctic stratosphere are consistent with observations of those species there, providing an important check on the proposed mechanism. Similar ozone changes are obtained with another possible heterogeneous reaction, H₂O + ClONO₂.

Recent satellite observations^{6,7} have revealed Antarctic ozone depletions over a very extended region in general agreement with the local ground-based data of Farman *et al.*¹. It was suggested¹ that the dramatic decreases may arise from a change

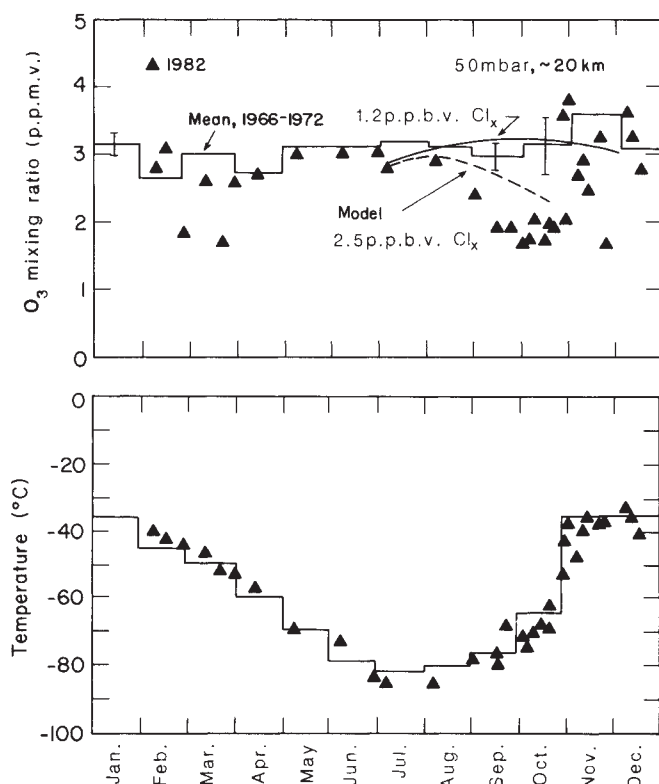
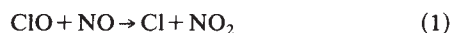


Fig. 1 Temporal behaviour of ozone mixing ratio at the 50-mbar level over Syowa, 69°S, compared with model calculations, for the late 1960s and early 1970s (histogram) and in 1982 ($\blacktriangle$)^{3,4}. Error bars represent standard deviations of the observations. Temperature observations for the same periods are also shown³.

in the NO_2/NO ratio due to the reaction



(and its relationship to ozone chemistry) which is of particular importance at the very low temperatures characteristic of Antarctic spring (-80°C). Clearly, this process should be expected to be of increasing importance with time as the chlorofluorocarbon content of the atmosphere increases due to anthropogenic releases. As Farman *et al.*¹ showed, reaction (1) can be effective in perturbing the ratio between NO and NO_2 near 30 km, and hence can influence ozone at those altitudes. However, the bulk of the ozone layer is located well below these levels in Antarctic spring, and so perturbations to ozone near 30 km can have only a modest influence on the total column (see Fig. 2 for a typical ozone profile). Most of the total ozone column at these latitudes and seasons resides near 15–20 km, where the ozone photochemical timescale is of the order of several years⁸ using presently accepted homogeneous photochemistry; it is at these low levels that a change must occur for the total column to be perturbed by such a substantial amount. Using a climatology of Antarctic winter temperatures presented by Labitzke⁹, we tested the role of reaction (1) in our dynamical-chemical two-dimensional model of the middle atmosphere^{10,11}. During the period from 1965 to about 1980 the total chlorine content of the atmosphere is estimated to have increased from about 1.2 to 2.5 p.p.b.v. (parts per 10^9 by volume)¹². This increase produced a 3–5% change in calculated ozone in Antarctic spring near 30 km, while the calculated changes at 20 km and below were less than 1%. The corresponding change in column-integrated ozone was, therefore, much smaller than that observed.

It is clear that further information regarding the vertical profile of the ozone perturbation is essential to any theory regarding

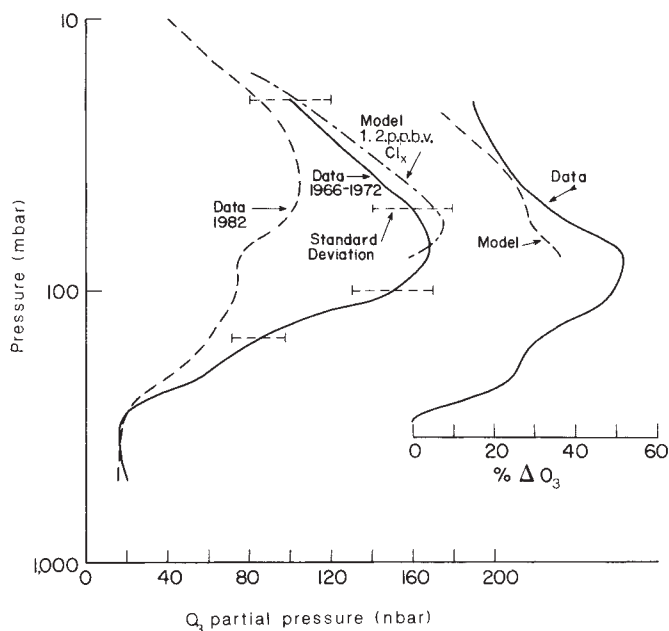
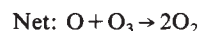


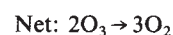
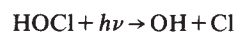
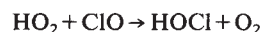
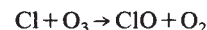
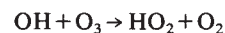
Fig. 2 Vertical profile of the ozone partial pressure observed over Syowa in the late 1960s and early 1970s, and the standard deviation, compared with observations in 1982, for the month of October^{3,4}. The model vertical profile in the late 1960s is shown for comparison. Percentage changes of the model and the data for the same time period are also indicated.

the ozone column change. We present below Antarctic balloonsonde data which show that the observed perturbation is indeed greatest at about 15–20 km, where it develops over a timescale of the order of 2 months. No significant changes in dynamical processes are indicated by the observed temperature structure there¹ (see also Fig. 2), suggesting that the cause of the ozone depletion is chemical rather than dynamical. If the observed depletions are the result of a chemical process, then that process must obey the following constraints: (1) it must be essentially confined to the Southern Hemisphere², (2) it must be a strong function of latitude and season; (3) it must produce an acceleration of the ozone chemical loss rate from a timescale of the order of several years to about a month near 15–20 km; and (4) it must increase with time over at least the past 10 yr. Constraint (4) suggests that we examine the photochemistry of atmospheric chlorine.

Chlorine can destroy ozone catalytically in the Earth's atmosphere through the following reactions:

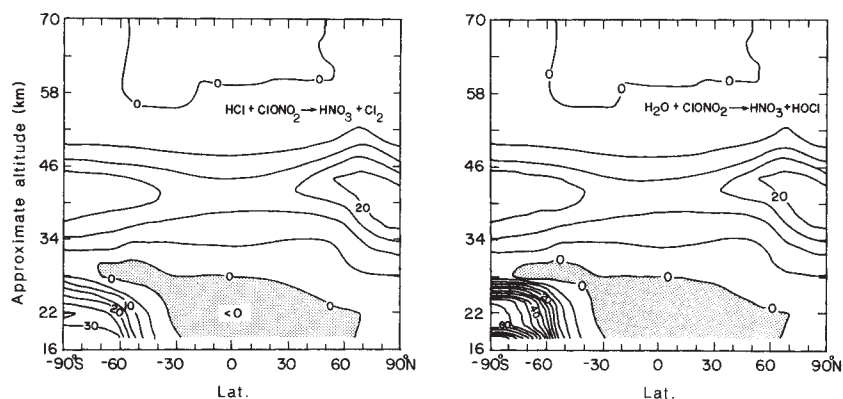


At lower altitudes, the following catalytic cycle should also be considered:



With present chemical schemes, the depletion due to increasing levels of atmospheric chlorine maximizes near 40 km, where much of the available chlorine resides as ClO and Cl. At lower levels, these catalytic free radicals are effectively converted to the 'reservoir' species, HCl and ClONO_2 , which are inert towards ozone. Both of these reservoirs are expected to be ~ 100 times

Fig. 3 Contour plot of the calculated change in ozone from the late 1960s to the early 1980s.



more abundant than ClO near 20 km, strongly inhibiting the Cl and ClO densities. Thus, if the densities of the catalytic species in the lower stratosphere are to be appreciably enhanced, both HCl and ClONO₂ must be severely reduced. One means of achieving this is the process



The Cl₂ will photolyse rapidly in the sunlit atmosphere, producing Cl. Laboratory studies suggest that reaction (4) does not occur rapidly in the gaseous phase, but is much faster on laboratory surfaces¹³⁻¹⁵. To alter the balances between the catalytic species and their reservoirs, which occur on a timescale of a few days, this reaction must proceed in Antarctic spring on a comparable timescale, requiring an equivalent two-body reaction rate of $\sim 2 \times 10^{-14} \text{ cm}^3 \text{ s}^{-1}$. Farman *et al.*¹ suggested that this reaction could be an alternative explanation for their observations, but they did not consider the need for a very rapid rate of this process in order to perturb the equilibrium between ClO and its reservoirs following the return of sunlight. Since reaction (4) is heterogeneous, it presumably takes place only in the presence of stratospheric aerosol, and indeed, the stratospheric aerosol surface area increases substantially in the vicinity of polar stratospheric clouds. These clouds are very common in the cold environment of Antarctic winter and early spring near 10–20 km, where their occurrence frequency is far greater than in the warmer Northern Hemisphere. They are observed predominantly between about July and September, and their presence is closely coupled to the temperature structure. The mean 1- μm aerosol optical depth in Antarctica is about an order of magnitude greater in winter and early spring than it is either during other seasons in Antarctica, or during any season in the Arctic⁵, suggesting that reaction (4) should be enhanced specifically during Antarctic winter and spring.

Figure 1 shows balloonsonde temperature and ozone observations from Syowa station, Antarctica (69°S, 39°E), at the 50 mbar (20 km) level³ (for the complete dataset, see the tape available from Ozone Data for the World). There are several years of historical data covering the period from about 1966 to 1972; the monthly mean of these data is shown as the histogram. In recent years, the station has been operated much less frequently, and virtually the only data available are those of an intense measurement programme carried out in 1982. Chubachi⁴ has shown that large changes in ozone were observed during this period, similar to those obtained by Farman *et al.*¹. The temperatures obtained at this particular station in 1982 were in general agreement with the historical data, while the ozone exhibited a dramatic departure in September and October.

Figure 1 also shows the results of our model calculations for 1.2 p.p.b.v. and for 2.5 p.p.b.v. including the hypothetical reaction (4) at an equivalent two-body rate as indicated above, imposed uniformly over the region from the model lower boundary at 100 mbar (about 16 km) to 26 km, for latitudes from

61°S to the Pole. The calculated seasonal trend in the unperturbed case, and the timescale of the calculated perturbation, are in reasonable agreement with those observed at the 50 mbar level. Calculated daytime-averaged ClO mixing ratios are about 1.8 and 0.4 p.p.b.v. at this level in September for 2.5 and 1.2 p.p.b.v. total chlorine (respectively), compared with ~ 0.02 and 0.01 p.p.b.v. when reaction (4) is not considered. Note that although the clouds are expected to be present in July, no ozone perturbation is observed or predicted then. This is consistent with the notion that the perturbation is photochemical, because the sunlight required to drive the photochemistry is present only after about August, when polar night ends at this latitude. Because this chemical perturbation occurs more rapidly (timescale of about a month) than do transport processes in this region (timescale of more than a season), this model can be used to study the problem even at levels near to the lower boundary at 100 mbar without significant bias due to the boundary conditions imposed^{10,11}. As mean temperatures rise above -80°C in October, the polar stratospheric clouds disappear⁵; the ozone remains low throughout October, but returns rapidly to levels much closer to the historical pattern in early November. This recovery is probably dominated by the acceleration of wave-driven transport accompanying the final spring warming of the Antarctic stratosphere, which brings in ozone-rich air from higher altitudes and lower latitudes. Because the behaviour of planetary waves cannot be calculated explicitly with our model, we are unable to simulate this rapid ozone increase.

Figure 2 presents the vertical profile of the October monthly mean of the observed ozone at Syowa for the historical years, as well as that obtained in 1982, along with the associated percentage changes. The observed changes are largest near 15–20 km, where they approach 40%. Figure 2 also shows the vertical profile of ozone obtained in our model for 1.2 p.p.b.v. chlorine and the percentage changes obtained for 2.5 p.p.b.v. when reaction (4) is considered. The vertical profile of the calculated ozone depletions, which agrees well with that observed, is determined by the chemical lifetimes of HNO₃ and ClONO₂. The equilibrium between ClO and ClONO₂ is established very rapidly if much NO₂ is present, and the ratio between the reservoir and the catalytic agent, ClO, is then so large that it will effectively inhibit the densities of Cl and ClO. Reaction (4) sequesters virtually all of the NO₂ in HNO₃, and the slow photolysis rate of HNO₃ at low levels implies that NO₂ cannot be released quickly enough to appreciably reduce ClO. At higher levels, where the HNO₃ photolysis rate is faster, NO₂ is released more rapidly and ClONO₂ formation effectively inhibits the ClO density. Note that large abundances of ClO can occur only if NO₂ levels are extremely low; thus the production of the HNO₃ product in reaction (4) is as important to the overall mechanism as the reactants.

Reaction (4) is not the only possible heterogeneous process capable of perturbing ozone. Indeed, another reaction that is

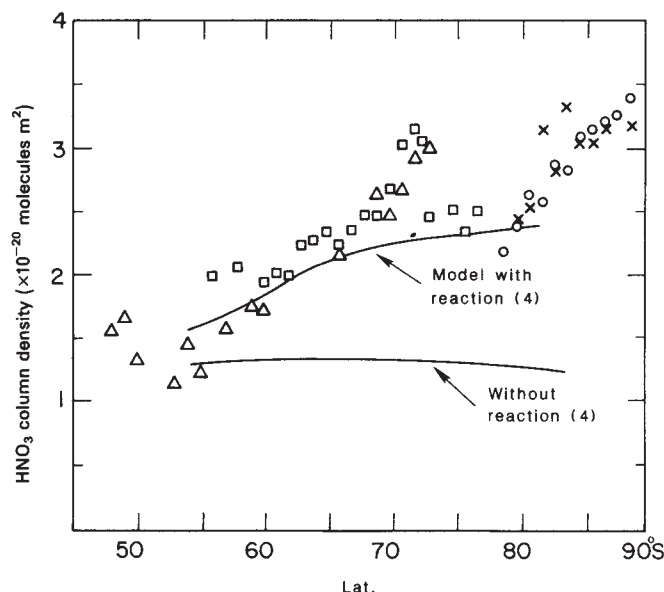
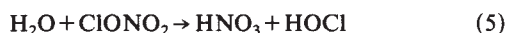


Fig. 4 Latitudinal gradient of total HNO_3 column abundance observed in Antarctic spring¹⁷, along with model calculations with and without consideration of reaction (4). $\square$, 17 November 1978; $\circ$, 20 November 1978; $\times$, 21 November 1978; $\triangle$, 27 November 1978.

similar to reaction (4) in its effects on the balance between ClO and its reservoirs, as well as in its effects on HNO_3 (if it proceeds at an equivalent two-body rate of $\sim 1 \times 10^{-17} \text{ cm}^3 \text{ s}^{-1}$) is



Laboratory studies show that this process also occurs heterogeneously¹⁵. Figure 3 shows a contour plot of the percentage ozone changes calculated between about 1965 and the early 1980s for both reactions (4) and (5). The high-altitude changes and their structure are due to standard chemistry⁸, but should be considered upper limits because of the neglect of the important effects of coupled temperature and CO_2 perturbations¹⁶. The calculated latitudinal structure in the additional depletions in the Antarctic lower stratosphere is due to the assumption that the clouds occur only poleward of about 60°S , confining the perturbation to high latitudes and low altitudes. A substantial gradient in mean cloud intensity (and hence in ozone depletion) probably occurs over the latitude range $60\text{--}90^\circ\text{S}$ because of the strong gradient in temperature over this region, but this has not been included in the present calculations. In the case of reaction (5), the possible production of HOCl leads to hydrogen radical formation, and thus produces even greater ozone depletions than those due to chlorine free radicals alone. The efficiency of reaction (5) in ozone destruction is thus critically dependent on HOCl as a reaction product.

Because HNO_3 is a product of both reactions (4) and (5), observations of odd nitrogen species provide an independent check on the feasibility of these processes. Figure 4 presents HNO_3 column abundance measurements from Antarctica in November¹⁷, along with model-calculated column abundances with and without consideration of reaction (4); reaction (5) produces similar results. Reaction (4) significantly improves the agreement between the model and the observations. Examination of the chemistry and transport of HNO_3 at high latitudes in Northern Hemisphere winter by Austin *et al.*¹⁸ reveals other evidence for an additional source of HNO_3 beyond presently accepted photochemistry, and points out that it is likely to be heterogeneous. This enhancement in HNO_3 should be accompanied by a decrease in other forms of active nitrogen (such as NO_2 and N_2O_5) in the lower stratosphere below about 25 km where the HNO_3 photolysis rate is slow, as discussed above with regard to the vertical extent of the ozone depletion. This implies that the total NO_2 column, and its diurnal variation in the spring, should be smaller in the Antarctic than in the Arctic due to the far greater frequency of polar stratospheric clouds in the Antarctic. McKenzie and Johnston's¹⁹ observations in September at 78°S display a sunset total NO_2 column of $\sim 1.5\text{--}2 \times 10^{15} \text{ cm}^{-2}$, with a diurnal variation of $< 0.5 \times 10^{15}$, while those of Noxon *et al.*²⁰ in March at 71°N suggest a sunset total abundance of $3\text{--}3.5 \times 10^{15}$, and a much larger diurnal variation of about $\sim 2 \times 10^{15}$, consistent with this suggestion.

Reaction (4) is certainly very speculative, and has been modelled only crudely here. However, it is consistent with all of the available features of the observed depletion of ozone, namely its vertical, latitudinal and temporal structure, as well as with observations of HNO_3 and NO_2 . The process responsible for Antarctic ozone depletion is probably heterogeneous, and related to odd nitrogen chemistry. In view of these considerations, as well as the lack of direct laboratory data regarding the kinetics and mechanism of reaction (4), we emphasize that other possible heterogeneous reactions, such as $\text{H}_2\text{O} + \text{ClONO}_2$ and $\text{H}_2\text{O} + \text{N}_2\text{O}_5$, should also be the subject of laboratory and atmospheric studies. Further observations of polar stratospheric clouds, and in particular, their occurrence frequency, are also badly needed. If the mean temperatures in Antarctic spring decrease (or have perhaps already decreased) by even 1 or 2°C , it is likely that the cloud frequency will increase, with associated changes in the aerosol surface area and rates of heterogeneous processes. Observations of ClO , or the more easily observable species, NO_2 , near Antarctic polar stratospheric clouds would be of considerable value in evaluating the mechanism responsible for the ozone depletion there. If heterogeneous reactions are indeed the cause of the Antarctic ozone phenomenon, these large depletions will be confined essentially to this region in the near future unless there is a very large increase in stratospheric aerosol.

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Reductions of Antarctic ozone due to synergistic interactions of chlorine and bromine

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The vertical column density of ozone observed in October over Antarctica has fallen precipitously over the past 10 yr. The concentration at Halley Bay (76° S 27° W), expressed conventionally in Dobson units (DU) (1 DU = 10⁻³ atmos. cm = 2.7 × 10¹⁶ molecules cm⁻²), has dropped from about 300 DU in 1975 to <200 DU in 1984 (ref. 1). Values in 1985 were even lower, comparable with the lowest values recorded anywhere on Earth². We suggest here that the loss of O₃ in Antarctica may be attributed to catalysis of O₃ recombination by a scheme in which the rate-limiting step is defined by the reaction ClO + BrO → Cl + Br + O₂. Concentrations of NO₂ must be low and heterogenous reactions involving particles in the polar stratospheric clouds must be an important element of the relevant chemistry. Industrial sources make important contributions to the contemporary budgets of both BrO and ClO and are likely to grow significantly in the future.

The decline in O₃ at Halley Bay does not simply reflect a local anomaly. It extends over at least 15° in latitude, according to measurements by the Total Ozone Mapping Spectrometer (TOMS) on Nimbus 7 (ref. 3). It is associated generally with air within the polar vortex, in a region offset some distance to the east with respect to the pole. The drop in O₃ begins in early September. The rate of decline in September 1983 averaged ~0.6% per day (ref. 3). The largest reductions in O₃ occur between 14 and 24 km, as evidenced by soundings from Syowa (69° S, 40° E)^{2,4}. The challenge for models is to account for the rate of change in September, and to rationalize the surprisingly short time constant implied by the observational data. It is particularly difficult to explain the large reductions observed at low altitude. A complete theory should account for the trend since 1975.

Several profiles adopted from ozonesonde data taken at Syowa^{2,4} are shown in Fig. 1. Curve *a* reflects the mean data for October before 1975. Curve *b* shows the average observed for the winter of 1982, while curves *c-e* illustrate conditions with much lower O₃ observed in October of 1982 and 1983. Temperatures were low for cases *c-e*, suggesting a significant contribution to the overhead column at these times due to air drawn from within the vortex. As noted above, reductions in O₃ are most striking near the peak at 70 mbar.

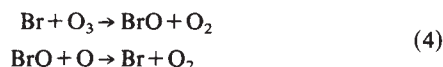
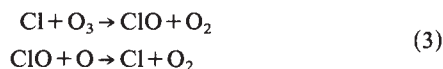
Ozone may be removed either by reaction with O,



or by reaction with itself,



These reactions proceed, for the most part, by indirect paths, catalysed by trace quantities of HO_x, Cl_x, Br_x and NO_x. Reaction (1) can occur through⁵⁻⁸



and

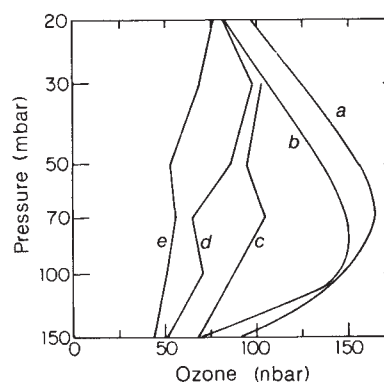
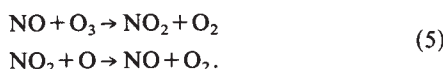
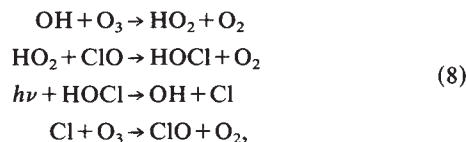
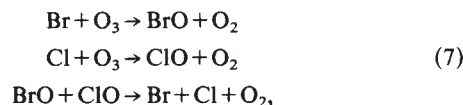
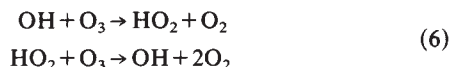
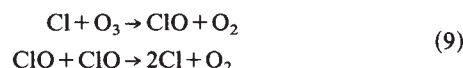


Fig. 1 Vertical distributions of ozone at Syowa (69° S, 40° E). *a*, An average of 26 soundings made between 1968 and 1974, in October. The average column density recorded during this period was 337 DU. *b*, An average of soundings made in June, July and August, 1982; the average ozone column was 320 DU. *c*, *d* and *e* were measured on 23 September 1982, 14 October, 1982, and 17 October, 1983; the ozone columns were 256, 241 and 217 DU, respectively. The mean ozone column for October 1982 was 236 DU; 260 DU for October 1983.

Reaction (2) can proceed by^{9,10}



and



If we assume that the change in Antarctic O₃ should be attributed to effects of chlorine, we can estimate the maximum possible impact from reactions (3)–(9), as follows.

Assume that Cl_x is present mainly as ClO and consider a profile for ClO corresponding to a total stratospheric chlorine abundance of 3.0 p.p.b. (parts per 10⁹), as would be appropriate for 1983. The associated (maximum) rate for removal of O₃ by reactions (3)–(5) is too small by an order of magnitude to account for the reductions implied at lower altitudes by the perturbed profiles of Fig. 1. The abundance of O at low Sun angles characteristic of high southern latitudes in September is insufficient to permit an important role for reaction (1) below 26 km.

No such restriction applies for reaction (2). In contrast to reaction (1), reaction (2) allows for significant loss of O₃ near 70 mbar. If we assume that chlorine and bromine are present primarily in the form of the reactive oxides, ClO and BrO, and consider concentrations of ClO and BrO of 3 p.p.b. and 25 p.p.t. (parts per 10¹²) at high altitude, we find that the column abundance of O₃ could fall by >2% per day, a rate significantly larger than observed by TOMS³.

High concentrations of ClO and BrO in the lower stratosphere require that the abundance of NO₂ be small. At mid latitudes the concentrations of NO₂ are high enough for ClO and BrO

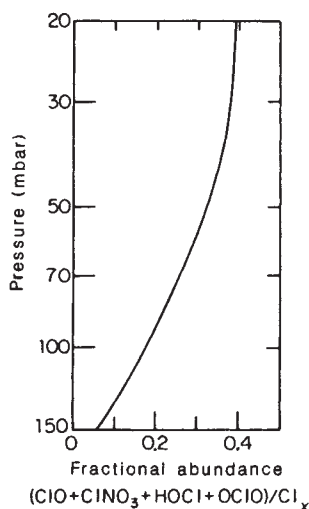
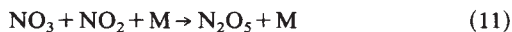
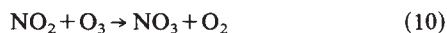


Fig. 2 Vertical distribution of the fraction of Cl_x present as ClO_x ($\text{ClO}_x = \text{ClO} + \text{ClONO}_2 + \text{HOCl} + \text{OCIO}$). The vertical profile of Cl_x was calculated at 30°S using a one-dimensional model, and Cl_x was estimated at 45° , 60° and 80° using slant mixing surfaces²⁵⁻²⁷. The fractional abundances of ClO_x were then calculated for the four latitudes using observed concentrations of ozone and a solar declination of 0° . The results of the four calculations are shown averaged along slant mixing surfaces approximately parallel to the tropopause. All calculations employed the rate constants recommended in ref. 22.

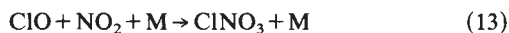
to be efficiently converted to ClONO_2 and BrNO_3 respectively. The abundance of NO_2 is very low at high latitudes in winter¹¹⁻¹⁴ and this is usually attributed to an influence of heterogeneous chemistry^{15,16} involving particles in clouds which form during the polar night^{17,18}. A plausible scheme is



and



Removal of ClONO_2 is possible also by¹⁹

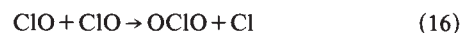


or by



Reactions (12), (14) and (15) have been observed on the walls of reaction vessels in the laboratory²⁰⁻²². Reaction (15) is unlikely to occur in the atmosphere in that it requires an interaction of two minor species present simultaneously on particles composed primarily of H_2O and H_2SO_4 .

The high-latitude stratosphere is coupled in autumn to lower latitudes by mixing along slant surfaces oriented roughly parallel to the tropopause^{23,24}. The average abundance of ClO_x (defined as $\text{ClO} + \text{ClONO}_2 + \text{HOCl} + \text{OCIO}$) relative to HCl along these surfaces may be estimated using one-dimensional model studies as a guide²⁵⁻²⁷. A mean profile, obtained from an average along the slant mixing surfaces, is given in Fig. 2. This should provide an appropriate representation of air entering the polar vortex. We expect the abundances of ClO_x and BrO_x (mostly $\text{BrO} + \text{BrNO}_3$) to remain essentially constant throughout the polar night. A combination of ClONO_2 formed by reaction (13), HOCl produced by reaction (14) and OCIO derived from



and



should represent the dominant forms of reactive chlorine. The potential importance of reaction (17) in this context was recognized first by Tung *et al.*²⁸.

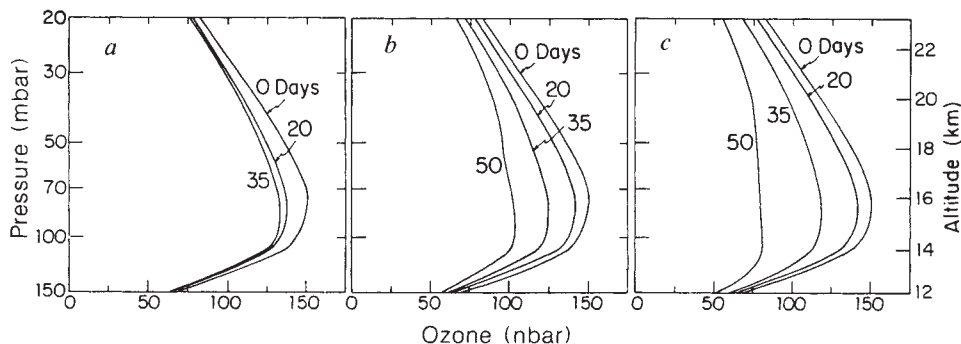
Consider two possibilities as the Sun returns to the high-latitude stratosphere in early spring: (I) Suppose that reactive chlorine is present mainly as OCIO . (The dominant source is reaction (17).) (II) Suppose that HOCl is more abundant. (This implies significant production by the heterogeneous reaction (14).) In either case BrO is likely to be the dominant form of reactive bromine. Assume that the initial abundance of NO_y (defined as all forms of reactive nitrogen other than HNO_3) is less than that of ClO_x .

In case I, OCIO provides an immediate source of ClO . The concentration of O_3 begins to fall, as illustrated in Fig. 3a. Removal is predominantly by reaction (7). Reduction in O_3 , and high concentrations of ClO and BrO , persist until the level of NO_y is comparable to that of ClO_x . Recovery of NO_y is regulated by decomposition of HNO_3 . The associated time constant for NO_y is about 10 days in mid-September.

In case II, photolysis of HOCl provides an immediate source of OH , in addition to Cl . If the subsequent chemistry were dominated by gas-phase processes, OH would react with HNO_3 leading to rapid growth in NO_y with conversion of ClO to ClONO_2 , short-circuiting the potential effect of reaction (7) as a sink for odd oxygen. A continuing role for reaction (7) requires that heterogeneous chemistry maintain a high ratio of ClO to ClONO_2 . We assume that reaction (14) proceeds with an

Fig. 3 Vertical distribution of ozone calculated for 20, 35 and 50 days after the end of polar night. The initial distribution of ozone was taken from Fig. 1b (320 DU). Chlorine and bromine abundances were 3.3 p.p.b. and 25 p.p.t. respectively, appropriate for 1985 (ref. 30). The initial fraction of Cl_x present as ClO_x was taken from Fig. 2; initial concentrations of NO_y were low, less than ClO_x . Calculations allowed for the diurnal variation of insolation, and ozone values were updated daily. Results are given for latitude 80°S .

a, Results for case I: ClO_x was assumed to be present as OCIO at the end of polar night, and the model allowed for gas-phase processes only. Results for 50 days are very similar to those for 35 days. b, Results for case IIa in which ClO_x was assumed to be present initially as HOCl , and the heterogeneous reactions (12) and (14) were allowed to proceed at a rate of $1.0 \times 10^{-5} \text{ s}^{-1}$. c, Results for case IIb, with a faster rate for reactions (12) and (14), $5.0 \times 10^{-5} \text{ s}^{-1}$. At 80°S , days 20, 35 and 50 correspond to 15 September, 1 October and 15 October. Model results indicate similar reductions in ozone one calendar week earlier at 75°S , two weeks earlier at 70°S .



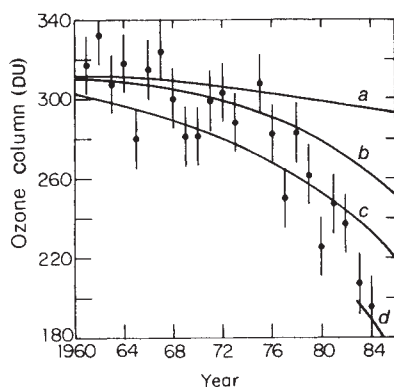


Fig. 4 Trend in the ozone column over Antarctica from 1960 to 1985. The circles correspond to October mean values from Halley Bay (76°S)¹. Results are given for 80°S , and assume an initial ozone column of 320 DU for each model year (see Fig. 3). The abundance of Cl_x was taken to be 1.0 p.p.b. in 1960, 2.1 p.p.b. in 1975 and 3.3 p.p.b. in 1985 (ref. 30); the abundance of Br_x was held constant at 25 p.p.t. *a-c*, Results for case I, IIa and IIb respectively, for day 50. *d*, Results for case IIb after 60 days. This curve illustrates the dramatic acceleration in O_3 loss which occurs if the aerosol burden persists.

accommodation coefficient of 2.5×10^{-2} and select an aerosol surface area appropriate for an extinction coefficient of $2 \times 10^{-3} \text{ km}^{-1}$. The associated reaction rate constant is $1 \times 10^{-5} \text{ s}^{-1}$. Extinction coefficients of $2 \times 10^{-3} \text{ km}^{-1}$ were typical for September in the Antarctic stratosphere before 1982 (ref. 17). Optical depths were much larger in 1983 (ref. 29) and 1984 following the arrival of the aerosol cloud associated with eruption of El Chichón (M. P. McCormick, personal communication). To account for additional aerosol loading, calculations were carried out also with a larger rate for reaction (14), $5 \times 10^{-5} \text{ s}^{-1}$. We shall refer to the low- and high-aerosol models as IIa and IIb. The polar clouds dissipate typically in late September when the aerosol burden is low¹⁷. They persisted longer in 1983 and 1984 when the aerosol burden was higher. The results in Fig. 3*b, c* should be interpreted accordingly.

The calculations summarized in Fig. 3 are appropriate for a latitude of 80°S . Sunrise at 80°S occurs at the end of August. Results for Syowa would be similar but the calendar date would be about 2 weeks earlier. With case I (Fig. 3*a*) there is a significant drop in O_3 over the first 2 weeks. The decline is slow thereafter, however, limited by the growth of NO_y and associated immobilization of ClO_x as ClONO_2 . The drop in O_3 persists longer if we allow for heterogeneous removal of ClONO_2 and N_2O_5 (Fig. 3*b, c*). The reduction at the peak, primarily due to reaction (7), is as much as 0.5% per day over the initial 30-day period if the rate constant for reaction (14) is as large as $5 \times 10^{-5} \text{ s}^{-1}$. The reduction exceeds 2% per day in the subsequent 15-day period, more than enough to account for the rate of decline observed by TOMS in 1983 (ref. 2). The faster decline at the later time is attributed to an increase in the abundance of ClO_x due to production of Cl by reaction of OH with HCl . If we allowed for heterogeneous removal of N_2O_5 and ClONO_2 , results for case I would agree with those for case II for times later than about 30 days.

Trends as a function of time are shown for total column O_3 in Fig. 4. The data, reflecting mean values for October at Halley Bay, are from ref. 1. As with Fig. 3, the model results are for a latitude of 80°S and are intended to illustrate the reduction expected 50 days after the end of polar night. The decline in O_3 would commence at the beginning of August in more northerly regions of the vortex. Effects would spread into the interior as the Sun advanced. The actual situation could be complicated by effects of transport, and precise comparison of model and

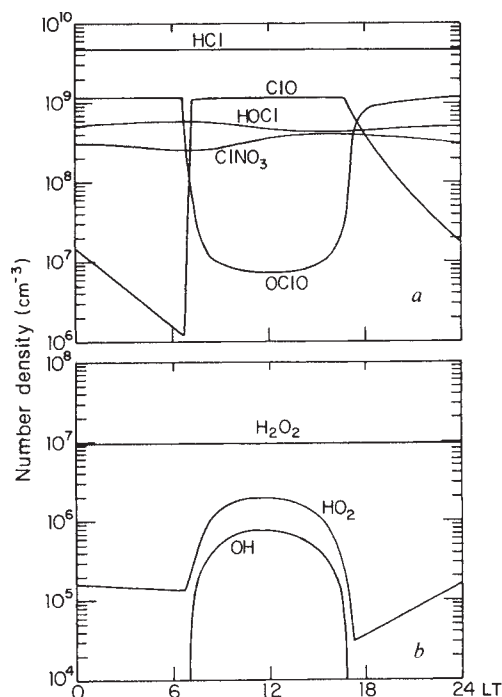


Fig. 5 Diurnal variations of Cl_x (*a*) and HO_x (*b*). Results are shown for case IIa, for day 20, 80°S latitude at 75 mbar ($\sim 16 \text{ km}$). The atmospheric density was $2.92 \times 10^{18} \text{ molecules cm}^{-3}$.

observational data is consequently difficult. The model results in Fig. 4 should be approximately applicable for mean conditions within the vortex at the beginning of October.

The model assumed an initial value of 320 DU for O_3 . The predicted year to year decline in O_3 at 50 days reflects the assumed growth of total chlorine, $4.7\% \text{ yr}^{-1}$ since 1973 (ref. 30). Case I, with only homogeneous processes considered, depicts a relatively small reduction in O_3 , about 25 DU in 1984. Reductions with case IIb should be as large as 100 DU for the same year. An intermediate effect is suggested for case IIa, 60 DU.

It is tempting to attribute the large reductions in O_3 observed in 1983 and 1984 to effects, both direct and indirect, of the aerosols from El Chichón. A reduction of 15% in O_3 was observed at northern mid-latitudes in winter and spring of 1983 (ref. 31), coincident with the time of maximum aerosol loading³². The reduction then could be associated with heterogeneous processes involving aerosols, enhanced perhaps by chlorine added to the stratosphere by the eruption³³. These effects could have an impact on Antarctic O_3 in addition to the $\text{ClO} + \text{BrO}$ contribution emphasized here and could contribute, at least in part, to the low values of O_3 observed since 1982.

The recombination scheme proposed here for O_3 is nonlinear, in the sense that the mechanism depends on the abundance, persistence and height distribution of aerosols. The rate-limiting step, reaction of ClO with BrO , involves a product of concentrations of two trace species each of which may be changing in time. The abundance of ClO is set mainly by decomposition of industrial sources of organic chlorine, notably CFCl_3 , CF_2Cl_2 and CH_2Cl_2 (ref. 5). The concentration of inorganic chlorine has increased steadily since 1960 (ref. 30). We assumed that the growth rate for inorganic bromine was comparatively small, and used a fixed concentration of 25 p.p.t. at high latitude in all calculations (ref. 27 and refs therein). There are reasons, however, to believe that the concentration of bromine may be increasing. Increases in production and use of CH_3Br , a fumigant, and CF_3Br and CF_2ClBr , employed extensively as fire extinguishers, are likely to have an important role in the future

course of stratospheric Br. Prospective levels could rise to as high as 100 p.p.t. (ref. 27).

The surprising results from Antarctica emphasize the potential importance and complexity of processes in the high-latitude stratosphere. Figure 5, for example, illustrates the diurnal variation expected for some of the dominant forms of Cl_x and HO_x . Observations are few and there is a need for additional data. Measurements of the abundance of aerosols, ClO , OCIO , HOCl , BrO , NO_2 and HNO_3 in winter and early spring would be valuable and would serve as a check on the model developed here.

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Temporal evolution of nitrogen compounds in Swedish precipitation since 1955

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Nitrate (NO_3^-) and ammonium (NH_4^+) ions are important determinants of the ionic strength and pH of precipitation¹. The deposition of these ions on vegetation and on the ground also constitutes a significant source of nutrients for plants. This is particularly the case in polluted regions, for example, Europe, where the deposition of nitrogen compounds is enhanced². There are indications (for example, increased run-off of nitrogen³) that forests in some parts of Europe have reached saturation levels with respect to nitrogen deposition; and excessive wet and dry deposition of nitrogen compounds has been suggested as a possible cause of the extensive damage to forests in Europe⁴. Data from Rothamsted, UK, indicate that the mean concentration of NO_3^- in precipitation doubled between the 1850s and 1960 (ref. 5); similarly, the NH_4^+ concentration probably increased between the 1880s and 1960. A review of precipitation chemistry data from Europe indicates an approximately threefold increase in NO_3^- deposition between the 1890s and the late 1970s⁶. The present analysis of data from the European Air Chemistry Network reveals that the NO_3^- concentration at most stations approximately doubled between the late 1950s and early 1970s, with a less pronounced increase in NH_4^+ . Detailed analysis of data from 12 Swedish stations showed no further increase of NO_3^- or NH_4^+ between 1972 and 1984. In contrast, the sulphate concentration decreased by ~30% for the same locations and time period.

The data that we have analysed are from the European Air Chemistry Network (EACN)^{7,8}. These data are obtained from monthly precipitation samples collected in bulk samplers at more than 50 stations in northern and central Europe since 1955. Details about the sampling and chemical analysis of ammonium

Table 1 Changes in concentration of NO_3^- and NH_4^+ in northern European precipitation between the late 1950s (median of all months 1955-59) and the early 1970s (1970-74)

Relative change %	No. of stations* (NO_3^-)	No. of stations* (NH_4^+)
> +250	3 (0)	1 (0)
+200-249	3 (0)	0 (0)
+150-199	6 (0)	3 (1)
+100-149	21 (5)	6 (0)
+50-99	20 (4)	12 (2)
0-+49	2 (0)	15 (3)
0-49	0 (0)	10 (2)
< -50	0 (0)	8 (1)
Total	55 (9)	55 (9)

* Numbers in parentheses refer to Swedish stations.

and nitrate ions as well as a listing of all the data between 1955 and 1979 are presented in refs 9 and 10, respectively.

Almost all (54 out of 55) EACN stations with sufficiently long records exhibit an increase in NO_3^- concentration between the late 1950s and the early 1970s. Table 1 shows the magnitude of the observed changes. At 75% of the stations the NO_3^- concentration increased between 50 and 150% during the 15-yr period, corresponding to an annual increase of 2-6%. Similar analyses have been published by Kallend *et al.*¹¹ and Horvath¹², who found an average annual increase of NO_3^- concentration at various sites to be ~6% and 7%, respectively. An increase in the NH_4^+ concentration was observed at 37 out of 55 EACN stations (Table 1). At half of the stations, the NH_4^+ concentration increased by 0-100%. Other previously published data¹² present no clear temporal trend of the NH_4^+ concentration between 1968 and 1981. Also, the variability in the NH_4^+ concentration was greater than that for NO_3^- . Note that the quality of some of the NH_4^+ data is dubious⁹, because microbiological activity may have an effect on the concentration of ammonium in the sample after collection.

For a more detailed analysis of temporal changes in nitrogen concentrations, we limited ourselves to the period between 1972 and 1984 and to Swedish stations only. We imposed this limitation mainly because this part of the network has been modified to meet more stringent sampling and analysis criteria¹³. For this period, data are available from 12 stations located in rural areas

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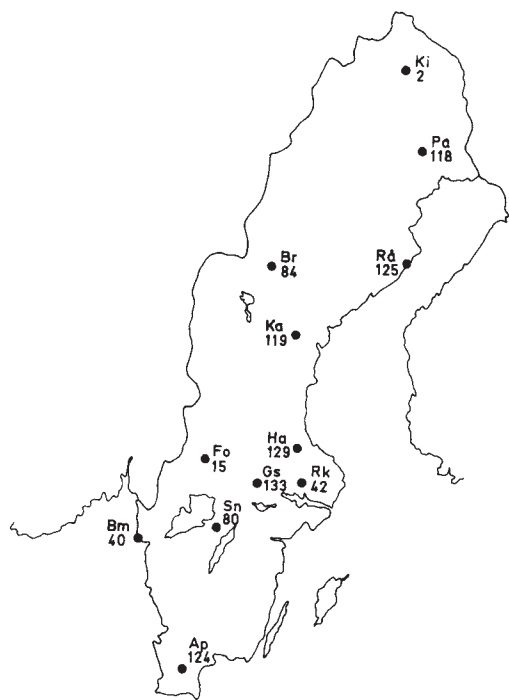


Fig. 1 Locations of 12 Swedish stations used in the statistical analysis.

and believed to be free of local sources of contamination (Fig. 1). Up to 1982, precipitation sampling was performed with 'bulk' collectors, that is with collectors that remain open during the entire month. Since 1981, sampling has also been performed with collectors that are used only during periods of precipitation ('wet-only' collectors).

Figure 2 shows the NO_3^- and NH_4^+ concentration in precipitation at individual stations located in northern (Br), central (Sn) and southern (Ap) Sweden. The annual values were obtained as precipitation-weighted averages of monthly values. A statistical filter was applied to remove outliers from the monthly values. This filter removed $\sim 1\%$ of the NO_3^- values and 4% of the NH_4^+ values. These stations were selected as examples because they have complete records and represent different geographical locations with different average concentrations. The general shapes of the time series from the other Swedish stations are similar. The values obtained by 'bulk' and 'wet-only' samplers agree quite well during simultaneous operation of both samplers (Fig. 2). No clear positive or negative trend in the nitrate or ammonium ion concentration was evident over the 13-yr period between 1972 and 1984.

To elaborate on the last point, we have performed linear regression analysis on data from all 12 Swedish sites. For NO_3^- , the slope was positive at five sites and negative at seven; at one of the sites, the slope was significantly (90% confidence level) greater than zero. For NH_4^+ , the slopes were also positive at five sites and negative at seven; with two of them being significantly different from zero (one greater than and one less than zero).

In contrast to the NO_3^- and NH_4^+ trends, the sulphate concentration showed a negative trend at all of the stations. Nine of the stations had trends significantly less than zero at the 90% confidence level. A decrease in sulphate concentration is consistent with decreasing anthropogenic sulphur emissions in several west European countries during the same time period¹⁴.

The temporal evolution of nitrogen oxides and ammonia emissions is not well established. In Sweden, the emissions of NO_x remained essentially constant between 1970 and 1983 (ref. 15). In France, FRG and the UK, NO_x emissions changed by +4, +15 and -3%, respectively, between 1975 and 1983

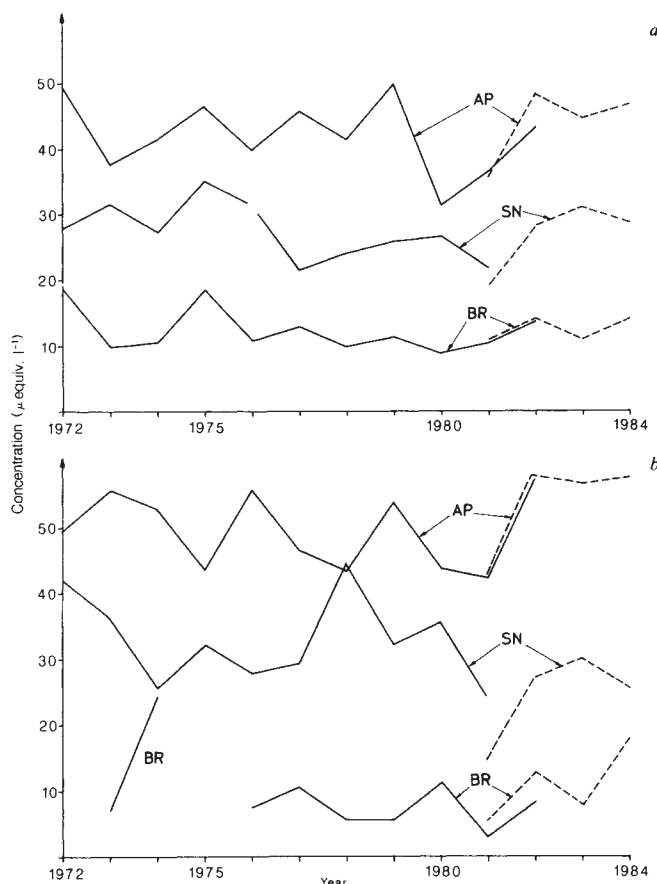


Fig. 2 Annual average concentrations of: a, NO_3^- ; b, NH_4^+ at three Swedish stations (shown in Fig. 1). 'Bulk' and 'wet-only' data are represented by continuous and broken lines, respectively.

(ref. 16). No reliable information seems to be available regarding changes in ammonia emissions.

The results presented here indicate a significant increase in the NO_3^- concentration in precipitation in northern Europe between the late 1950s and the early 1970s. However, since then no significant change has occurred in Sweden. A similar situation holds for the NH_4^+ concentration, although the increase from the late 1950s to the early 1970s was less pronounced than for NO_3^- .

Unless the forest damage that has been observed during the past few years in FRG¹⁷, and suspected to have occurred in Sweden¹⁸, is caused by an unlikely increase in dry deposition, by nitrogen deposition accumulated over several years or by infrequent events of very high concentration/deposition, it is hard to believe that only NO_3^- and NH_4^+ in precipitation are responsible for such damage. This conclusion is based on the lack of correlation during the past decade between the observed forest damage and the concentrations of these ions in precipitation.

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High-temperature fluid-rock interactions in a layered syenite pluton

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Variation in mineral composition with stratigraphic height in layered igneous intrusions is generally taken to indicate progressive fractional crystallization, and, following pioneering work on the Skaergaard complex¹, petrologists have used this variation in modelling the evolution of magma bodies. In cooling plutons, however, major chemical changes may occur in circulating deuteric fluids^{2,3}. In the Klokken (South Greenland) layered syenite we have discovered that mineral compositions vary both along and normal to the strike of individual layers, and we argue that major chemical changes have occurred in a circulating aqueous fluid constrained to move along relatively permeable high-temperature aquifers in the layered series.

The Klokken intrusion is a predominantly syenitic stock in the Gardar alkaline province in South Greenland (Rb-Sr age $1,139 \pm 11$ Myr; ref. 4), with an outer sheath of gabbro and an inner ring of structureless unlaminate syenite⁵ (Fig. 1). Current work shows that the unlaminated syenites become more evolved inwards, suggesting that they formed by congelation against the chamber wall. They enclose an elliptical chamber measuring 3×2 km, in which the more evolved layered syenite series formed. The exposed thickness is ~ 600 m; modelling using trace elements suggests that the chamber was $>1,320$ m thick at this stage⁶, and both the cryptic variation and studies of cryptoperthite exsolution textures show that the roof was close to the highest present levels of exposure⁷. The layered sequence is cut by a body of more basic biotite-syenodiorite (Fig. 1), which has the form of a feeder dyke which spreads laterally near the topographic summit of the intrusion to form a sheet.

The layered series dips inward at $30-50^\circ$, flattening close to a focus (F, Fig. 1a). Individual mineral layers may be traced for up to 3 km along strike. The layered series consists of two texturally contrasting syenite types⁵. Layers of fine-grained granular syenite form $\sim 15\%$ of the exposed section, and cryptic variation shows that these represent a roof-chill series, continuing the trend of the unlaminated syenite (Fig. 2a), which was stoped in a *lit-par-lit* fashion by the laminated syenites which form the remainder of the succession. These laminated syenites show repeated mineral layering of an unusual, inversely graded type (Fig. 3), in which normal leucocratic syenite gives way upward, characteristically over 2-3 m, to hedenbergite pyroxenite or fayalite-rock⁵. The layering is ascribed to variations in the crystal supply brought about by changes in nucleation and growth rates under a regime of fluctuating P_{H_2O} (ref. 5).

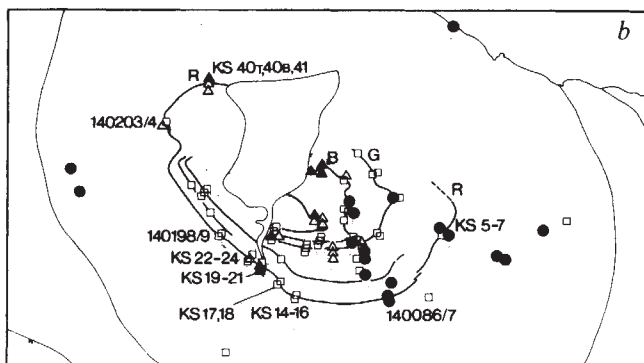
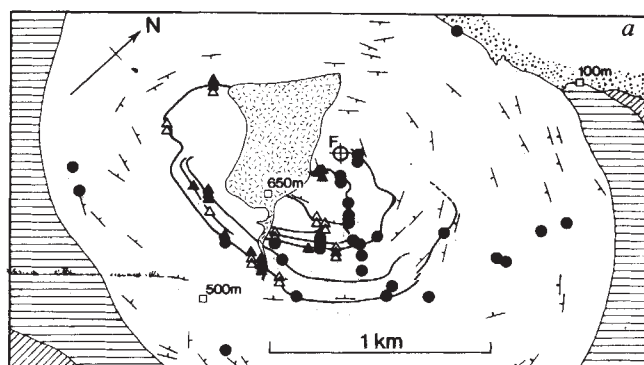


Fig. 1 a, Outline geological map of the central part of the Klokken complex. Oblique hatching, gabbro; horizontal hatching, unlaminated syenite; dashed, late syenodiorite. In the northern corner of the map the igneous rocks are obscured by glacial gravels. In the layered series the inward dips are shown ($30-50^\circ$), with a focus at F. Individual mafic layers in the laminated syenite series which have been traced on the ground are shown as heavy lines. Note that the topography falls steeply towards the north (see spot heights). Symbols show the per cent anorthite component ($\text{CaAl}_2\text{Si}_2\text{O}_8$) in the ternary alkali feldspars: $\bullet$, $<2\%$; $\triangle$, $2-3\%$; $\blacktriangle$, $3-8\%$. b, As a, but illustrating clinopyroxene variation in terms of the scheme shown in Fig. 2: $\blacktriangle$, high-Mg, field 1; $\triangle$, high-Mg, field 2; $\square$, normal, field 3; $\bullet$, high-Na, field 4. The layers B, G and R, which have been studied in detail, are labelled, as are samples from layer R used in the construction of Fig. 2.

The mineralogy of the Klokken layered series comprises alkali feldspar $\gg$ Fe-rich clinopyroxene $>$ Fe-rich olivine = ilmenomagnetite, with biotite, amphibole, quartz and sphene occurring as intercumulus phases only. The magmatic evolution of the complex is revealed most clearly by the pyroxenes, as summarized in Fig. 2a. The granular syenites become systematically more evolved downwards, and generally show considerable compositional contrast with the more evolved, interbedded laminated syenites. 70% of analysed clinopyroxenes in the laminated syenites plot close to the hedenbergite composition (field 3, Fig. 2b), showing that the liquid from which they crystallized was the end product of extended *in situ* fractionation, of which the granular syenites represent less evolved stages^{5,6}.

In detail the laminated syenite sequence is more complex. We collected along and normal to individual layers, selecting three (R, G, B in Fig. 1b) for detailed study. We also collected from other layers and from 'normal' massive syenite. Figure 2b shows the pyroxene variation along the outcrop of layer R; Fig. 3 shows the variation normal to the layer. (See Fig. 1b for sample localities.) Variation on a similar scale was found in the other layers studied. Clearly, intra-layer variation is as large as in the layered series as a whole. To show variation with locality, we have divided the diopside-hedenbergite-acmite triangle (Fig. 2b) into four fields, and classified each sample of laminated

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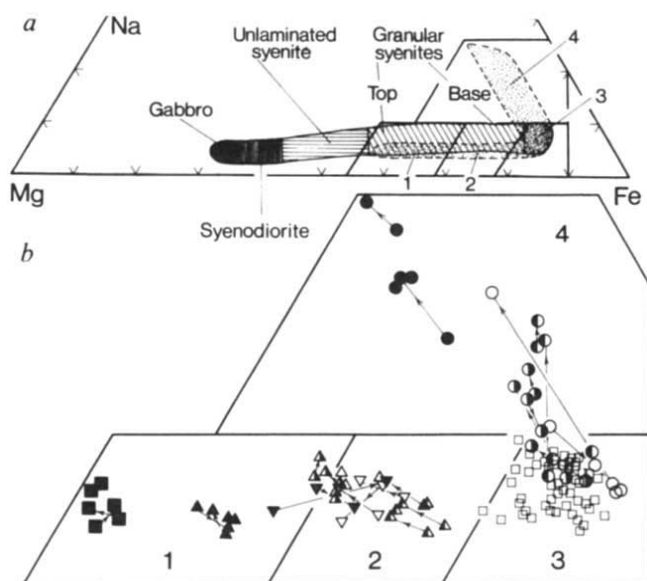


Fig. 2 *a*, Triangular diagram summarizing the range of clinopyroxene variation in all units of the Klokken intrusion (atom per cent Na, Mg and Fe calculated on the basis of 6 oxygens; $\text{Mg} = \text{CaMgSi}_2\text{O}_6$, diopside; $\text{Fe} = \text{CaFe}^{2+}\text{Si}_2\text{O}_6$, hedenbergite; $\text{Na} = \text{NaFe}^{3+}\text{Si}_2\text{O}_6$, aegirine). The stippled area shows the range of all laminated syenites; the majority (>70%) lie in the region of heavy stippling. *b*, Variation within a single layer (R) of the laminated syenite series. Fields 1–4 were used to classify high-Mg and high-Na clinopyroxenes as shown in Fig. 1*b*. Samples which fall predominantly in fields 1, 2 and 4 may be identified by their symbols (●, KS 6; ○, 140086; ○, 140087; ○, KS 7; ■, KS 19; ▲, KS 20; ▼, KS 23; ▲, KS 40B; ▲, 140204; ▽, KS 41; △, KS 40T), and their localities found in Fig. 1*b*. All other samples ('normal' laminated syenites) are shown as squares. Tie lines indicate zoned crystals, with arrows pointing towards rims.

syenite according to the field in which the pyroxene predominantly falls. Figure 1*b* shows the distribution of pyroxene analyses according to this scheme: high-Mg examples (fields 1 and 2) all come from an area south of the focus of the complex, in the vicinity of the late syenodiorite body; the most magnesian come from the sampling points nearest to the syenodiorite. The high-Na pyroxenes (field 4) come from the outer part of the layered series, irrespective of stratigraphic position. Figure 3 shows the relationship between pyroxene composition and lithology for points in layer R, arranged in order of distance from the syenodiorite dyke.

Other primocryst phases show similar variation. Layer G contains olivine in which the Mg/Fe ratio varies sympathetically with that of coexisting clinopyroxene. Subtle variation is found in the alkali feldspars, which have bulk compositions in the range $\text{Ab}_{75}\text{Or}_{25}$ – $\text{Ab}_{55}\text{Or}_{45}$, with up to 8 mol % An (ref. 7). All samples with >2% An come from the same broad region of the complex, offset from the focus, in the vicinity of the late syenodiorite. Those with >4% An come from localities within 50 m of the diorite, although not all such samples have high An. Away from this central area all samples have <1% An.

Conventional analysis of the cryptic variation would suggest that the outer part of the layered series crystallized from a more evolved liquid than the inner, in marked discordance with the mineral layering, and in an opposite sense to the inward evolution of the sheath of unlaminated syenite. The variation is not related in any direct way to stratigraphic height (Fig. 1), as is the case in the simplest layered intrusions¹, or to the focus of the layering, as might be expected if the discordance were the result of a lateral accretion process^{8,9} or of crystallization in a chemically zoned magma body¹⁰. Ponding of light magma beneath granular layers can be ruled out because the latter

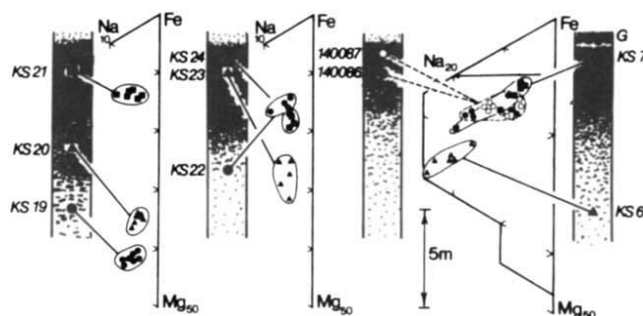


Fig. 3 Relationship between clinopyroxene composition and position relative to bedding in layer R. The layer, which is unusually thick, shows inversely graded mineral layering, with a diffuse top which grades upwards into normal syenite considerably more rapidly than downwards. The sections are arranged from left to right in order of distance from the syenodiorite dyke (Fig. 1). The shading shows the general change in feldspar/pyroxene ratio (light, feldspar; dark, pyroxene) in the inversely graded layer; feldspar primocrysts are not to scale. At the locality of samples KS 5–7 a granular syenite layer (G) appears above the mafic horizon. Note that the most extreme compositions, both high-Mg and high-Na, tend to come from the more leucocratic parts of the layers.

become rare and discontinuous towards the margin of the layered series. The complex and variable relationship to lithology within individual layers (Fig. 3) also rules out all these hypotheses.

We suggest that the compositions of the main mineral species in the laminated syenite series have been modified, immediately after crystallization, in a large-scale deuteric fluid system. This system left the granular and unlaminated syenites largely unaffected. Textural evidence consistent with this hypothesis is the reversed zoning observed in the high-Mg pyroxenes (Fig. 2*b*) and the normal zoning (Na replacing Ca) exhibited by the high-Na examples. In thin section the zoning is patchy, being related to fractures as well as grain boundaries. The most extreme examples of high-Mg pyroxene and high-Ca feldspar come from close to the syenodiorite and we can see no other explanation for this than high-temperature, sub-solidus modification. Exsolution microtextures in the cryptoperthitic alkali feldspars⁷, indicating exsolution by spinodal decomposition at >850 °C in the most An-rich examples, show that Ca exchange occurred at even higher temperatures, before exsolution. Patchy zoning in the alkali feldspars has been demonstrated by transmission electron microscopy, as well as by electron microprobe⁷.

Field evidence of the presence of high-temperature, alkaline solutions in the outer part of the layered series is provided by a suite of drusy peralkaline pegmatites which occur as lenses and veins at the boundary between the layered series and the unlaminated syenites. The development of a pegmatitic facies of the laminated syenite in flame structures at the base of granular layers and the presence of druses in the laminated syenites demonstrate the presence of a fluid phase during the final stage of crystallization^{11,12}.

Persistence of deuteric fluids down to lower temperatures is demonstrated by the turbid alteration of the alkali feldspars¹¹, which are white and translucent in the laminated syenites, and transparent, often dark green, in the granular and unlaminated syenites. The turbidity is related to the development of patch perthites in place of strain-controlled braid crypto- or micro-perthites, which occurred at <450 °C (ref. 13). The most turbid samples tend to be those containing the most Na-rich pyroxenes, and these frequently also contain replacive alkali amphibole and aegirine. Extensive replacement of magmatic phases by hydrous phases at low temperatures, as is found in the Scottish Tertiary epigranites², is not found in Klokken. Granular syenite feldspars have retained radiogenic ⁴⁰Ar, while the laminated

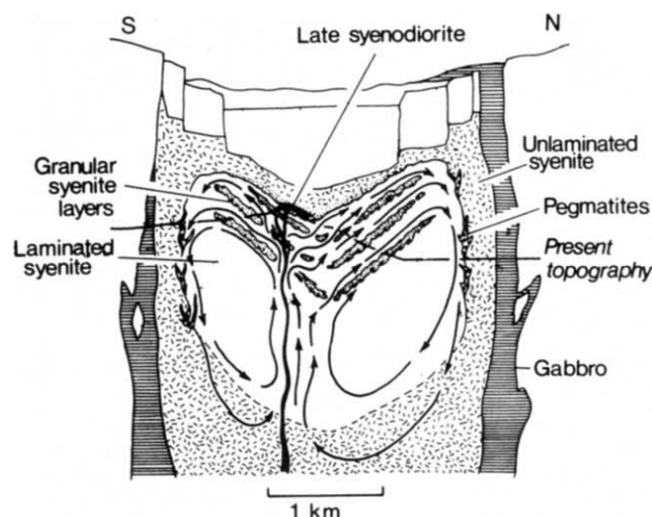


Fig. 4 Stylized section through the Klokken chamber showing suggested flow-lines (arrows) for the deuteritic fluid and the disposition of the main rock units. Although the thickness of the layered series (laminated + granular syenites) is approximately correct⁴ (equal horizontal and vertical scales), for clarity the thickness of the granular layers is greatly exaggerated (real thickness 0.1–30 m) and their number underestimated (there are ~40 granular layers in the exposed section, and they become thicker and more abundant upwards)^{5,12}. The caldera superstructure is speculative.

syenite feldspars have leaked argon in proportion to their turbidity¹⁴. We conclude that the coarse-grained, drusy laminated syenite layers acted as high-temperature aquifers in the layered complex, over at least the temperature range 850–450 °C, probably corresponding to a cooling interval of $>10^5$ yr (ref. 13). The relatively fine-grained, compact-textured granular layers acted as impermeable horizons. Variable permeabilities account for the irregular relationships observed across layers at single localities (Fig. 3). Generally, the mafic parts of inversely graded layers have feldspars which are less turbid than those in leucocratic parts, suggesting that the mafic layers were less permeable than the leucocratic syenite.

Fluid circulation has been modelled numerically for the Skaergaard intrusion¹⁵, which water is thought to have entered from the well-jointed basalt envelope. In contrast, the Klokken layered series was sealed against the ingress of water by the outer gabbro and unlaminated syenite rings (Fig. 4). Biotites in the gabbros form a series with OH/F ratios different from those in all syenites, suggesting that they were in equilibrium with a different fluid (I.P., R. A. Mason and S.M.B., in preparation).

Although the late syenodiorite intrusion strongly influenced the mineral chemistry in its vicinity, it seems unlikely that this comparatively small body would have provided the heat source necessary to drive kilometre-scale convection in the entire chamber. The summit sheet is thin, and the feeder dyke is only ~3 m wide at its intersection with layer R (Fig. 1). We envisage an initially symmetrical convecting system upon which the thermal effect of the late-arriving syenodiorite was superimposed (Fig. 4), causing a local perturbation in the flow regime.

The roof of the complex was close to the present level of erosion⁷, and Fig. 4 shows an axial rise of heated water from the comparatively slowly cooling interior; this water is then constrained to move laterally along inclined laminated syenite aquifers beneath the roof. Pyroxenes in equilibrium with this fluid were more Na- and Fe-rich in the cooler, outer part of the system, and in the inner core the mineralogy of the layered series was modified by the emplacement of the more basic syenodiorite. Klokken is unusual in that its laminated syenites crystallized from a residual magma which was at or near water-

saturation⁵. Large-scale modification of mineralogy may be unlikely in more basic intrusions. We note that the discordant mineral variation found in the Skaergaard intrusion¹⁰ occurs at the junction between the Upper Zone and the Upper Border Group, a situation analogous to the granular syenite/laminated syenite boundary at Klokken. Although infiltration metasomatism has been postulated as a post-cumulus source of mineral modification in the Muskox intrusion¹⁶, this is a local re-equilibration with intercumulus silicate liquid. It is improbable that the effects at Klokken could result from kilometre-scale circulation of intercumulus melt, particularly in view of the relatively low temperature and high silica content of the residual liquid. Circulation of residual melt is also unlikely because individual layers tend to have distinctive intercumulus assemblages (for example, quartz is found only in certain layers, as is intercumulus sphene).

In the Klokken intrusion, sub-solidus modification of the consolidated sequence by deuteritic fluids appears to have produced compositional variation very similar to that usually ascribed, in layered complexes, to crystal-melt fractionation. Although most salic plutons are not layered, they almost invariably exhibit low-temperature deuteritic alteration, and by analogy with Klokken, substantial changes in mineral chemistry may also accompany the circulation of fluids at high temperatures during their cooling history.

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Jack Hills, evidence of more very old detrital zircons in Western Australia

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The age of the Earth's oldest crustal minerals sets a time-limit on the earliest preservation of buoyant solid crust. The oldest mineral ages reported so far are ~4,180 Myr for detrital zircons from quartzites at Mount Narryer¹, in the Yilgarn Block, Western Australia. The oldest-known intact rocks, as distinct from individual minerals, are substantially younger; they formed ~3,813-Myr ago² in the Isua supracrustal belt, West Greenland. We report here a further occurrence of old detrital zircons, again identified using the ion-microprobe SHRIMP³, in conglomerate from the Jack Hills Metasedimentary Belt^{4,5} (26°11' S, 116°58' E),

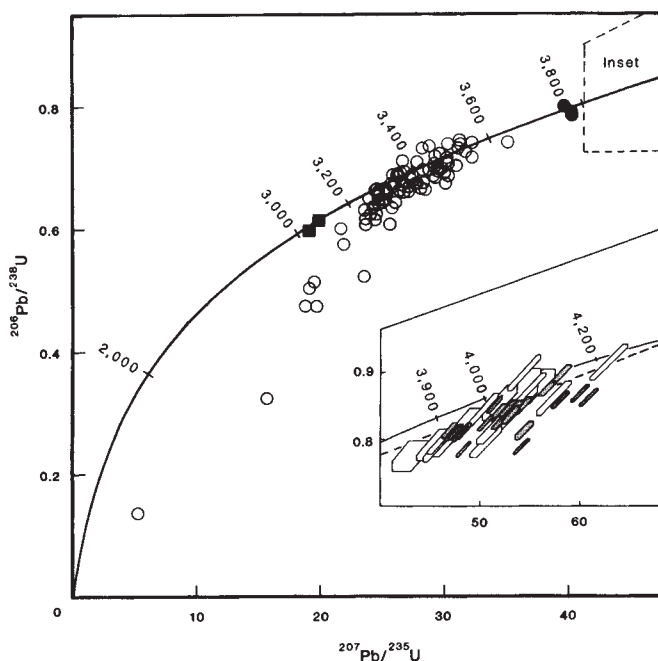


Fig. 1 Concordia diagram showing U-Pb ages by ion microprobe for 140 grains of detrital zircon from Jack Hills conglomerate sample W74. All points on the main diagram and the open symbols in the inset are reconnaissance analyses. The filled symbols in the main diagram denote concordant ages for the youngest and oldest grains in the younger population, and those in the inset are precise analyses. The inset shows the complete data (Table 1) for the 17 oldest grains. Uncertainties are one standard error. The broken line is a hypothetical early Pb-loss line to 3,500 Myr.

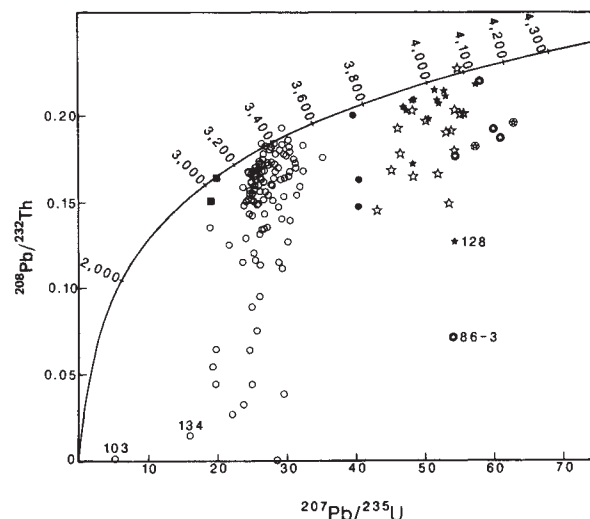


Fig. 2 Concordia-type diagram showing Th-Pb ages. Symbols are as for Fig. 1, except that analyses of the older crystals are shown as stars. ☆, reconnaissance; ★, precise analyses; circled stars, grain 86.

~60 km north-east of Narryer. This discovery is important for several reasons. First, one zircon registers the exceptionally old age of $4,276 \pm 6$ Myr, which is, moreover, a minimum estimate for its original age; 16 other grains may have the same age or may be slightly younger. Second, the new occurrence considerably extends the area over which the old Narryer suite is known, thus amplifying its geological significance. Third, the frequency of old zircons in the Jack Hills occurrence is 12%, about five times higher than at Narryer, which suggests that the intact ~4,180 Myr-old rocks, if they still exist, might be closer, and facilitates future comparison of the SHRIMP data with conventional analyses of these grains. (Recent conventional U-Pb work⁶ on other zircons from the same Narryer concentrate confirmed the younger ages but failed to observe any at ~4,180 Myr, probably because of their low abundance⁷.) The likelihood that zircon would dissolve in magmas undersaturated in zirconium⁸ argues against its survival in long-term contact with such magmas during the disaggregation and subduction of crust. Consequently, the existence of zircon in the Jack Hills area from ~4,300 Myr ago implies that this part of the crust has been preserved, since then, from recirculation in the mantle through plate-tectonics or any other mechanism.

The Jack Hills Metasedimentary Belt is a narrow belt ~70 km long composed of minor metabasalts and thick chert and banded iron formation interleaved with clastic metasediments⁵. It is broadly similar to the nearby Narryer Metamorphic Belt⁹ but lower in metamorphic grade (high greenschist). It is separated from surrounding granites and gneisses by zones of major deformation which prevent direct observation of the relative ages of the granitic rocks and the belt. Some of the granites have been dated using conventional zircon U-Pb techniques as ~3,500 Myr (gneissic granite) and ~2,600 Myr (undeformed granite)⁵. The detrital zircons were concentrated from a chert pebble conglomerate within a thick clastic sequence in the southwestern section of the belt. They vary from almost colourless to deep purplish

brown, and are predominantly broken (probably during processing) and rounded. Most of the latter are pitted suggesting transport abrasion. Rare grains have a blunted prismatic form. Only a few are structured into a core surrounded by a distinct rim of younger zircon. Several analysed grains show faintly the euhedral internal zoning that is generally considered to result from magmatic crystallization, but none of the 4,100–4,300-Myr-old zircons (hereafter termed the 'old' zircons) have this feature. Most of the zircons are devoid of inclusions although some, including three old grains, contain a few randomly oriented, transparent, acicular inclusions. The 17 old zircons identified cannot be distinguished on colour or morphological criteria from the other rounded grains.

The U-Pb results are plotted in Fig. 1, which shows that the analyses of most zircons lie either within error of Concordia or slightly below it, signifying only small losses in their uranogenic Pb. In contrast, a few areas within grains have lost much of their radiogenic Pb comparatively recently. For concordant zircons and those having Pb loss at zero-age only, the original ages are calculable from the radiogenic $^{207}\text{Pb}/^{206}\text{Pb}$. Where the Pb-loss history is more complex, the $^{207}\text{Pb}/^{206}\text{Pb}$ ages can be interpreted reliably as minimum estimates for the original ages. (We emphasize that Pb isotopic ratios are directly measured without peak-stripping on SHRIMP and are independent of standard comparisons and calibrations; as such they provide straightforward minimum age-determinations for each analysed area.) The $^{207}\text{Pb}/^{206}\text{Pb}$ age for most grains is ~3,350 Myr, which we interpret as their age of crystallization or high-grade metamorphic recrystallization before deposition in the conglomerate. Those between 3,400 and 3,600 Myr (Fig. 1) probably represent older zircons that formed at different times within that interval, and there are several grains that formed earlier still at ~3,800 Myr. Two zircons are distinctly younger than all the rest with concordant ages at just less than 3,100 Myr. They are detrital, having rounded forms and abraded surfaces, and their age constrains the deposition of the conglomerate to later than that value. The $^{207}\text{Pb}/^{206}\text{Pb}$ ages that exceed 3,900 Myr belong to a much older population which may have had a single original age close to 4,300 Myr and have undergone early as well as recent Pb loss, or it may be a mixed-age population that formed during discrete events over an extended time period from 4,100 to 4,300 Myr ago.

The details of reconnaissance and precise analyses of the 17 old zircons are listed in Table 1. The five analysed areas within grain 86, which shows the highest minimum ages, exhibit small

Table 1 Ion microprobe analyses for U, Th and radiogenic Pb for $25 \times 35 \mu\text{m}$ areas within the older zircons from Jack Hills

Grain-area	U (p.p.m.)	Th (p.p.m.)	% Common 206*	$\frac{208}{232}$	$\frac{206}{238}$	$\frac{207}{235}$	$\frac{207}{206}$	Minimum age (Myr)
22-1R	49	25	3.4	0.180 ± 23	0.880	54.4	0.448 ± 8	
22-1P	56	24	0.6	0.172 ± 6	0.784	48.3	0.446 ± 2	$4,072 \pm 7$
31-1R	55	22	4.2	0.166 ± 26	0.854	51.9	0.441 ± 7	$4,055 \pm 24$
34-1R	217	113	1.0	0.197 ± 8	0.843	50.2	0.432 ± 3	
34-2P	82	34	0.6	0.195 ± 6	0.824	50.4	0.444 ± 2	$4,065 \pm 7$
43-1R	39	27	9.7	0.149 ± 27	0.857	53.6	0.453 ± 12	
43-1P	46	33	1.4	0.206 ± 7	0.826	51.9	0.456 ± 3	$4,105 \pm 10$
46-1R	57	23	4.4	0.165 ± 27	0.821	48.4	0.427 ± 8	$4,007 \pm 28$
51-1R	70	47	3.4	0.178 ± 14	0.797	46.5	0.423 ± 7	
51-1P	71	47	0.2	0.209 ± 5	0.808	47.9	0.430 ± 2	$4,017 \pm 7$
53-1R	32	20	7.2	0.145 ± 33	0.774	43.1	0.403 ± 13	$3,920 \pm 48$
56-1R	149	95	0.6	0.227 ± 9	0.873	54.9	0.456 ± 3	
56-1P	160	104	0.3	0.214 ± 9	0.846	52.7	0.452 ± 2	$4,092 \pm 7$
82-1R	39	25	3.2	0.201 ± 19	0.878	55.6	0.459 ± 8	
82-1P	47	30	0.7	0.211 ± 6	0.836	53.0	0.460 ± 3	$4,118 \pm 10$
86-1R	96	58	2.5	0.182 ± 10	0.861	57.4	0.483 ± 4	
86-1P	115	70	0.5	0.220 ± 5	0.859	58.0	0.490 ± 2	$4,211 \pm 6$
86-2R	174	152	1.0	0.196 ± 6	0.915	62.9	0.499 ± 3	
86-2P	187	156	0.5	0.192 ± 5	0.868	60.0	0.501 ± 2	$4,244 \pm 6$
86-3P	181	327	0.9	0.071 ± 2	0.789	54.1	0.497 ± 2	$4,232 \pm 6$
86-4P	113	87	0.5	0.187 ± 4	0.862	60.9	0.512 ± 2	$4,276 \pm 6$
86-5P	40	20	2.1	0.175 ± 8	0.811	54.4	0.485 ± 4	$4,196 \pm 12$
92-1R	59	26	3.3	0.169 ± 19	0.791	45.1	0.414 ± 6	$3,961 \pm 22$
102-1R	174	77	1.4	0.203 ± 9	0.899	54.4	0.439 ± 3	
102-1P	165	76	0.1	0.215 ± 3	0.852	51.4	0.438 ± 2	$4,045 \pm 7$
119-1R	186	108	0.7	0.193 ± 6	0.801	46.1	0.418 ± 3	
119-1P	198	112	0.2	0.205 ± 3	0.812	46.9	0.418 ± 2	$3,975 \pm 7$
119-2P	182	102	0.5	0.209 ± 4	0.840	51.8	0.447 ± 3	$4,075 \pm 10$
P	177	95	0.1	0.209 ± 4	0.812	48.3	0.431 ± 3	$4,021 \pm 10$
123-1R	61	24	1.9	0.190 ± 18	0.828	52.4	0.459 ± 6	$4,115 \pm 19$
123-1P	70	28	0.8	0.225 ± 10	0.898	58.2	0.470 ± 3	$4,150 \pm 9$
	71	28	0.5	0.212 ± 8	0.877	56.8	0.470 ± 3	$4,150 \pm 9$
128-1R	77	23	2.3	0.182 ± 21	0.816	51.2	0.455 ± 5	$4,102 \pm 16$
128-2P	93	53	1.0	0.123 ± 5	0.850	54.3	0.463 ± 3	$4,128 \pm 10$
	101	54	0.4	0.130 ± 5	0.845	53.4	0.458 ± 3	$4,111 \pm 10$
130-1R	114	41	1.0	0.193 ± 12	0.789	45.7	0.420 ± 4	$3,982 \pm 14$
130-1P	123	44	0.6	0.201 ± 7	0.810	47.2	0.423 ± 3	$3,993 \pm 11$
	127	45	0.3	0.205 ± 5	0.811	47.5	0.425 ± 2	$4,000 \pm 7$
138-1R	91	57	2.3	0.180 ± 11	0.796	50.5	0.461 ± 5	$4,121 \pm 16$

Analytical procedures are described elsewhere². Uncertainties shown are one standard error and refer to the last digits only. Coefficients of variation are 1.5% for precise Pb/U, 3.0% for Th/U and reconnaissance Pb/U, and 10% for U, Th concentrations.

R, Fast reconnaissance analysis using three scans.

P, Precise analysis using 14 scans and lower content of surface-related common Pb.

* Surface-related common Pb, taken as Broken Hill ore; sample 206/204 is 1600/(% common 206).

but real differences in radiogenic $^{207}\text{Pb}/^{206}\text{Pb}$ that can only have been generated by internal redistribution or loss of radiogenic Pb relative to U at some early time. The slightly discordant distribution of U–Pb data within this grain and all except possibly one of the other old grains (Fig. 1) is similar to that observed in many zircon suites in Archaean rocks elsewhere, and is customarily explained by minor Pb loss during one or more later events. Nevertheless, another conceivable explanation involves an early gain of Pb (or loss of U) followed by recent Pb loss, which would have the effect of increasing the $^{207}\text{Pb}/^{206}\text{Pb}$ age of the zircons. Redistribution of Pb that resulted in such 'reverse' discordance (points above Concordia) has been reported¹⁰ for three out of seven areas analysed in one exceptional zircon from an orthogneiss at Mount Sones (Napier Complex, Antarctica), but the phenomenon of reverse discordance is very rare. In view of this and the overall consistency of the slightly (normally) discordant data for the old zircons, such a complex and *ad hoc* mechanism involving reverse discordance followed by recent Pb loss is considered most unlikely as an explanation of the observed old U–Pb ages. Grain 86 also shows large differences in Pb/U between areas having the same $^{207}\text{Pb}/^{206}\text{Pb}$,

which indicate recent Pb loss. Because there have been two or more apparently independent Pb loss events, a single Pb loss vector in Fig. 1 cannot be constructed for grain 86 to estimate its original age of crystallization. All that can be said is that $4,276 \pm 6$ Myr, the highest $^{207}\text{Pb}/^{206}\text{Pb}$ age listed in Table 1, is most likely a minimum estimate for the original age.

Additional evidence for recent Pb loss from many of the zircons, including grain 86, is shown in Fig. 2, a Concordia plot of $^{208}\text{Pb}/^{232}\text{Th}$ against $^{207}\text{Pb}/^{235}\text{U}$. Many of the analysed zircons are close to Concordia on this diagram, but assuming no U or Th movement, many others have lost ^{208}Pb relative to ^{207}Pb . The reliability of Th/U and Pb/U as measured by SHRIMP has been documented elsewhere³, so that the differential loss of Pb isotopes (which has also been seen elsewhere¹¹) is a property of particular areas of zircon. The timing of this loss is equal to or younger than the youngest observed $^{208}\text{Pb}/^{232}\text{Th}$ age, which within error is zero. None of the data fall above Concordia, indicating that only Pb losses are registered rather than both gains and losses. The area showing the greatest loss of ^{208}Pb , 86-3, also has the lowest $^{207}\text{Pb}/^{235}\text{U}$, and the other zircons that are most discordant in Pb/U such as grains 134 and 103, also

have the lowest $^{208}\text{Pb}/^{232}\text{Th}$. A plausible hypothesis is that all the old zircons first formed at $\sim 4,300$ Myr, then lost Pb during one or more early events such as the known felsic magmatism at $\sim 3,500$ Myr and the uplift and weathering of the zircon provenance at $\sim 3,100$ Myr. Their present compositions would lie on the hypothetical chord shown in Fig. 1 if no Pb had been lost subsequently. Instead, because Pb loss also occurred recently, they are variably scattered below it.

In the absence of other evidence, we have attempted to use the internal structure, morphology and Th, U contents of the detrital zircons to suggest the nature of their original source rocks. The nearly universal rounding of the zircons is ambiguous, as we cannot ascertain how much of the rounding is due to mechanical abrasion and how much to metamorphic corrosion. The most important genetic features of the 140 analysed zircons are the lack of internal structure and the lack of inclusions in most grains, the blunted euhedral forms of some zircons, and the faint euhedral zoning in the rest. Their mean U, Th, at about 100 and 50 p.p.m. respectively, is distinctly low relative to the average crustal zircon, and zircons with similar morphological features and with low U and Th contents are known in only a few rock types. Zircon populations made up almost entirely of coarse, rounded, uranium-poor, transparent, inclusion-free, unzoned zircon occur in pyroxene granulites¹². Consequently, we favour mafic rocks that experienced granulite facies metamorphism as the probable sources for most of the detrital zircons from the Jack Hills conglomerate. In contrast to the old zircons from Jack Hills, all but one of those from Narryer have distinc-

tive subhedral forms and pronounced euhedral internal zoning. The Narryer zircons also have significantly higher uranium, but not thorium, contents. These differences suggest that the zircons in the Narryer quartzite came from felsic igneous rocks not recrystallized by granulite grade metamorphism, and provide evidence for considerable geological complexity in the ancient terrain contributing zircons to the two metasedimentary belts.

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Carbon-14 dates point to man in the Americas 32,000 years ago

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The view that man did not arrive on the American continent before the last glaciation has been supported by the fact that until now the known and dated archaeological sites have not been of very great antiquity. But now we report radiocarbon dates from a Brazilian site which establish that early man was living in South America at least 32,000 years ago. These new findings come from the large painted rockshelter of Boqueirão do Sitio da Pedra Furada, the walls and the ceiling of which are decorated with a rich set of prehistoric paintings. We have excavated a sequence containing abundant lithic industry and well-structured hearths at all levels. Carbon-14 dates from charcoal establish a continuous chronology indicating human occupation from $6,160 \pm 130$ to $32,160 \pm 100$ years BP. A date of $17,000 \pm 400$ BP, obtained from charcoal found in a level with fragments of a pictograph fallen from the walls, testifies to the antiquity of rupestrian art in this region of Brazil.

The site of Boqueirão of Pedra Furada is one of the 200 known painted rock-shelters located in a remote region of the Brazilian plateau famous for its richness in prehistoric rupestrian art (Fig. 1). This site, discovered in 1973 by the French-Brazilian archaeological mission in Piauí, is a rock-shelter situated on the steep bank of a 100-m non-calcareous sand-stone cliff, standing 20 m above the bottom of the valley of Pedra Furada.

The first excavation campaign at the site of Boqueirão of Pedra Furada was undertaken to date the rupestrian art which is abundant in the region, and the first two dates obtained were from $7,640 \pm 140$ yr (Gif-4928) and $8,050 \pm 170$ yr BP (Gif-4625)¹.

The excavations were extended in area and depth during the following years. In 1985, the bedrock was finally reached after more than 3 m of sediment had been excavated.

Traces of human occupations succeed one another throughout the stratigraphic sequence. Based upon the distribution and number of artefacts which were in any case associated with the hearths, it appears that the site was occupied only by a small human group on temporary basis. The first traces of human presence in the shelter are a few scattered pieces of charcoal and two lithic pieces, found in the lowest layer A. These were a large pebble bearing the scars of several flakings, and a flake. This lowest level could not be dated.

All of the sedimentary layers contained remains which can be classified, according to the lithic industry and the type of structures of the hearths, in two main stages called Pedra Furada and Serra Talhada². The artefacts unearthed in the surface layer E are different from those found in the lower layers, but not characteristic enough to permit definition of a cultural phase.

During the most ancient cultural phase, Pedra Furada, large circular hearths are found. They are well built with fallen blocks and contain large quantities of charcoal and ash. Lithic industry is abundant and localized around the hearths. The raw materials used for the fabrication of tools were quartz and quartzite, from the pebbles present at the site. Based upon typological and archaeological criteria, this cultural phase has been divided into four stages. In the earlier stage, Pedra Furada I, a lithic collection composed of 560 pieces was found, principally pieces with blunt points obtained by two, three or four convergent flakings, made of pebbles or flakes. Besides these typical tools, pebble-tools (chopping tools and choppers) appear as well as denticulates, burins, notched pieces, retouched flakes and double-edged flakes (Fig. 2).

In addition to the retouched pieces, the collection includes pebble-hammers, flaked pebbles, flakes and fragments produced by flaking. In this level, fragments of painted rock, fallen from the walls, are perhaps evidence of the ancient practice of rupestrian art. Two dates, $31,700 \pm 830$ yr (Gif-6652) and $32,160 \pm 1,000$ yr (Gif-6653) have been obtained from charcoal coming from the first hearth found at the site.

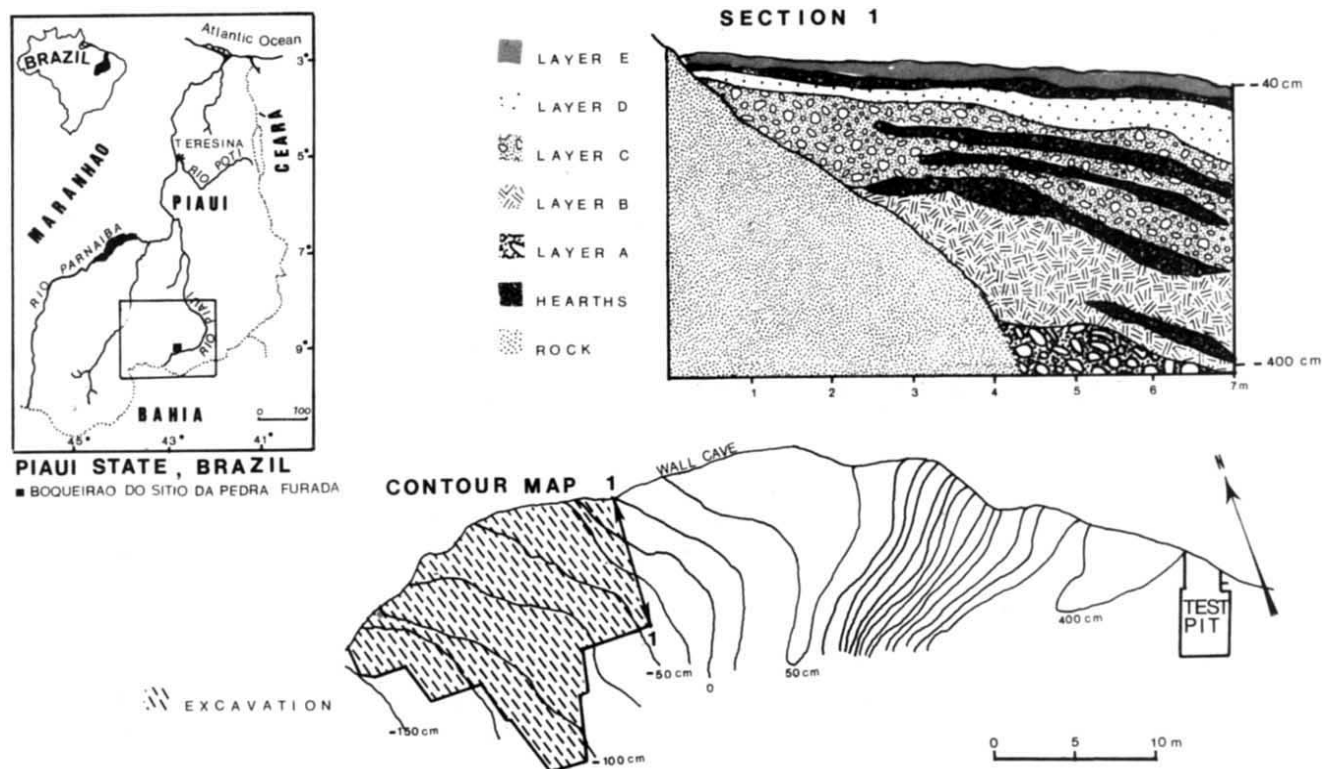


Fig. 1 The shelter 'Toca do Boqueirao do Sitio da Pedra Furada' is situated in the state of Piauí, in northeastern Brazil. This area, known by archaeologists as São Raimundo Nonato, is one of the most important in America, with 244 sites discovered there: 186 painted shelters, 13 painted and engraved shelters, 10 sites with only engravings, and 1 site with fossil megafauna. Five distinct sedimentological layers are distinguished, from the bottom to the top: **A**, beige-coloured sand containing coarse grains (≥ 1 mm): 5% pebbles and silt: 8.5 to 10.5%. The sediment is compact. **B**, compact beige-pink sand with silt: 6 to 8% and lower percentage of coarse grains. Pebbles are more abundant in some places. **C**, pinkish sand with silt, 4 to 6% and with gravels and pebbles in some places. **D**, brown to beige coloured sand with coarse grains (≥ 1 mm): 8% and gravels and pebble-beds in some places. This layer is rich in organic matter, mostly ash and charcoal. **E**, at the surface, brown-grey coloured layer with composition identical to that of the lower layers. It is very loose and contains numerous organic remains. The layers B, C, D and E contain saucers or lenses-shaped hearths characterized by a heavy concentration of ashes and charcoal.

Table 1 Carbon-14 dates in years BP and correlation between divisions and sedimentological layers for the site of Pedra Furada

Layers	Occupations		Chronology	Rock art
Layer E	Last occupations		Relative: 5,000 BP	Agreste tradition
Layer D	Phase Serra Talhada	IV Final Stage	6,160 $\pm$ 130 BP (GIF 5863)	Nordeste tradition
		III Late Stage	7,750 $\pm$ 80 BP (GIF 6161)	
			7,640 $\pm$ 140 BP (GIF 4928)	
Layer C	Phase Pedra Furada	II Middle Stage	8,050 $\pm$ 170 BP (GIF 4625)	Fragments of pictographs spalled from the walls
		I Early Stage	8,450 $\pm$ 80 BP (GIF 6162)	
			Relative: 10,000–12,000 BP	
		IV Final Stage	17,000 $\pm$ 400 BP (GIF 5397)	
Layer B		III Late Stage	21,400 $\pm$ 400 BP (GIF 6160)	Painted fragments spalled from the walls
Layer A	First occupations	I Early Stage	23,500 $\pm$ 390 BP (GIF 6158)	Painted fragments spalled from the walls
			$\geq 25,000$ BP (GIF 5648)	
			$\geq 25,000$ BP (GIF 5398)	
			25,200 $\pm$ 320 BP (GIF 6147)	
			26,300 $\pm$ 800 BP (GIF 6309)	
			26,400 $\pm$ 500 BP (GIF 5962)	
			27,000 $\pm$ 800 BP (GIF 6308)	
			29,860 $\pm$ 650 BP (GIF 6651)	
			31,700 $\pm$ 830 BP (GIF 6652)	
			32,160 $\pm$ 1,000 BP (GIF 6653)	

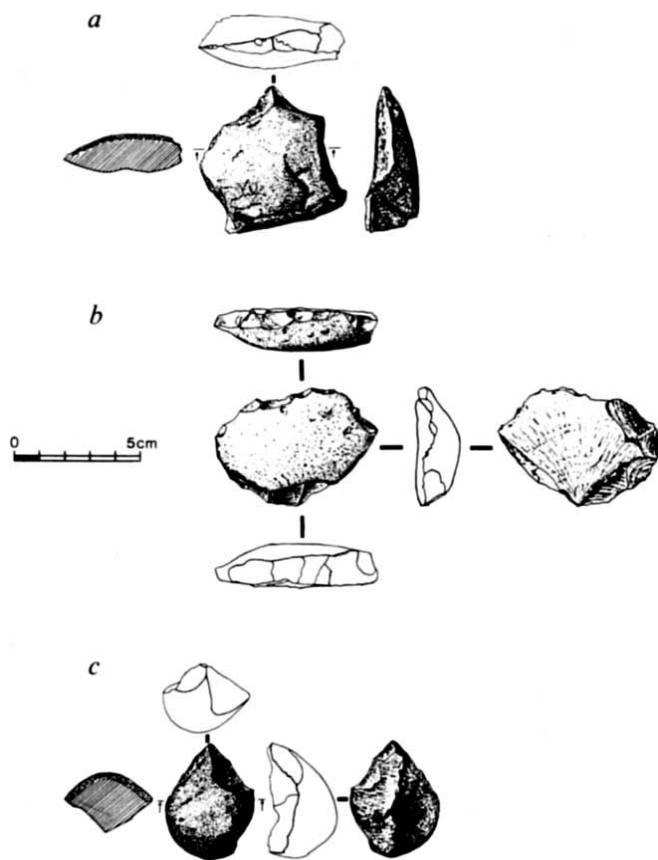


Fig. 2 Large quantities of lithic artefacts were recovered at Pedra Furada I. The lithic pieces are performed by unifacial retouching with a hard hammer although some bifacial pieces are present. *a*, A notched point made from quartz flake; the retouch on edges is flat and regular. *b*, *c*, Blunt points obtained by 2, 3 or 4 convergent flakings. Blunt points are made from flakes or pebbles, they show some traces of utilization.

The next stage, Pedra Furada II, produced 711 lithic pieces. Eight different hearths which correspond to eight distinct periods of occupation have been dated, giving ages from $23,000 \pm 390$ yr (Gif-6158) to $29,860 \pm 650$ yr (Gif-6651) (Table 1). Hence the site Pedra Furada was occupied at different times and probably on a temporary basis from 32,000 to 23,000 yr BP. This occupation is supported by the presence of numerous hearths and the abundant evidence of lithic industry.

The characteristic tools of Pedra Furada III are the blunt points made out of pebbles or flakes, along with chopping-tools, denticulates, knives, retouched and unretouched flakes, and pebbles bearing flaking scars. This stage was dated at $21,400 \pm 400$ yr (Gif-6160). A new series of occupations which represents the final stage of Pedra Furada is characterized by side-scrapers and blunt points. The other retouched pieces are small bifaces, denticulates and double-edged tools.

A carbon-14 date was obtained for one of the hearths and gave the age of $17,000 \pm 400$ yr (Gif-5397). A piece of rock with two red painted lines was found in the same hearth. This pictograph indicates the practice of rupestral art at that time and makes the site of Pedra Furada the most ancient rupestral art site known in America, and one of the most ancient in the world.

The upper layers of the site have produced artefacts which belong to another cultural unit, Serra Telhada: flint and siltite appear little by little and replace quartz, as the raw material for tools, the retouching of the tools is of better quality, and the

hearths are of different types. The absence of hearths and charcoal have precluded the dating of the beginning of this phase. An age of between 10,000 and 12,000 years BP can be suggested based on analogy with lithic material found in a test pit in section E of the site which gave an age of $10,400 \pm 180$ yr (Gif-5862).

Higher in the stratigraphic column, a level of dense occupation is found. The hearths are surrounded by pebbles and blocks and the base covered by a slab, showing traces of successive utilizations. The lithic industry is quite varied and the flaking of blades is an innovation. The proportion of pieces of flint is important even though quartz and quartzite continue to be employed. Retouched pieces include end-scrapers, side-scrapers, knives, blunt points, planoconvex retouched blades and flakes, chopping-tools, bifaces, notched-pieces and pebble-hammers.

Ochre, which is present in large quantities in this level, as well as fragments of painted rock, suggest that rupestral art has been an important activity during this period, which has been placed between $8,050 \pm 170$ (Gif-4652) and $8,450 \pm 80$ yr (Gif-6162). Subsequently, the hearths become larger and more diversified in their structure. Towards the end of the phase Serra Telhada, they become elliptical in outline. Rich in ashes and charcoal, they also contain fragments of bone, wood, leaves, and fruit pits. Nearby are found a large quantity of lithic objects such as flint end-scrapers of three types: discoidal, steep, and nosed. The end of this phase has been dated at $6,160 \pm 130$ yr (Gif-5863). The uppermost sediments are very disturbed by recent human activity and cannot be used for study.

Thanks to the abundance of charcoal present in almost all of the levels, the occupation of this site has been dated in a systematic manner. The seventeen carbon-14 dates obtained from charcoal samples collected during the excavation of the site, are summarized in Table 1. From bottom to top, their succession is not only internally consistent but also consistent with the stratigraphy. These dates, along with the archaeological remains discovered, make it possible to establish the sequence of the different cultural phases of the site. They also establish the antiquity of the human presence in the region. Most archaeological sites known at present in America are not very old. The most ancient ones, the stratigraphy and the dating of which have not given rise to controversy, are comparatively young: 13,000-yr-old for the site of Monte Verde in Chile³, 14,200-yr-old for the site of Alice Boer in Brazil⁴ and 19,450 yr for Meadowcroft Rockshelter in Pennsylvania⁵. Others, recently claimed as being older, are doubtful since the presence of man has not been proved, or since the methods used for dating are questionable. An example of questionable dates is provided by the very old dates of bones, obtained by aspartic acid racemization⁶. Holocene ages were produced for these same samples when measured by accelerator mass spectrometry⁷. The present findings for the site of Pedra Furada testify to the presence of man in the north of South America 32,000 years ago, and strongly suggest that the migration from Asia to North America occurred earlier.

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Synaptosomes possess an exocytotic pool of glutamate

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There is increasing evidence that L-glutamate is a major excitatory neurotransmitter in the central nervous system^{1,2}. Immunocytochemical studies indicate that glutamate within nerve terminals may be concentrated in vesicles³ and glutamate-accumulating vesicles have recently been isolated⁴. Exocytotic release of glutamate from synaptosomes (isolated nerve terminals) has not been convincingly demonstrated, however, and remains highly controversial⁵⁻⁷. In order to study the kinetics of release of endogenous L-glutamate from guinea pig cerebral cortical synaptosomes we have devised a continuous enzymatic assay. This has enabled us to identify a pool, equivalent to 15–20% of the total synaptosomal glutamate, which is capable of rapid Ca²⁺-dependent exocytotic release.

If synaptosomes are incubated in a medium containing glutamate dehydrogenase and NAD⁺, then release of the amino acid into the medium should cause an increase in fluorescence due to the formation of NADH. Figure 1 shows that this system has sufficient sensitivity to detect a release within seconds from less than 2 mg of synaptosomal protein. The advantages over existing techniques are considerable: detection is rapid and continuous; there is no need to chromatograph multiple samples to obtain a single time-course; complexities due to isotopic metabolism are avoided and errors due to incomplete isotopic equilibration between intrasynaptosomal compartments cannot arise.

Incubations of synaptosomes with polarized plasma membranes show a decrease in fluorescence once external glutamate has been assayed (Fig. 1a). The decrease is sensitive to rotenone and is due to the slow reoxidation of NADH by the small fraction of damaged mitochondria present in the incubation. Although it has been reported that synaptosomes degrade micromolar concentrations of added NAD⁺, any hydrolysis of the 1 mM NAD⁺ added was insufficient to affect the sensitivity of the assay to glutamate, even after a 10 min preincubation.

When Ca²⁺-depleted synaptosomes are depolarized by adding 30 mM KCl to the incubation medium, efflux of glutamate is negligible for the first 15–20 s, after which there is a slow efflux (Fig. 1b). When KCl is added in the presence of Ca²⁺, a much more rapid increase in fluorescence results (Fig. 1c). The fluorescence lags behind the actual glutamate release due to the finite activity of the glutamate dehydrogenase. This hysteresis may be quantified from the rate at which an added glutamate standard is assayed to completion and used to correct trace 1c. The correction is negligible for the slow release in the absence of Ca²⁺. The corrected trace (Fig. 1d) shows that the Ca²⁺-dependent component of glutamate release is complete by 30–45 s. In five experiments, the mean size of the rapid, Ca²⁺-dependent component of glutamate efflux was 1.83 ± 0.2 nmol mg⁻¹ ($n = 5$). For comparison, the glutamate released by complete permeabilization of the synaptosome with 0.05% Triton X100 was 11.0 ± 1.0 nmol mg⁻¹ ($n = 4$).

NAD⁺ inhibits evoked synaptic potentials in the guinea pig hippocampus⁸; however it does not appear to inhibit glutamate release from cortical synaptosomes because there is a good agreement between the release seen by the continuous assay in the presence of NAD⁺ (Fig. 1d) and the release within 30 s of 2.1 ± 0.09 nmol glutamate mg⁻¹ as determined by HPLC in the absence of NAD⁺ (unpublished data). The fluorometric assay is insensitive to aspartate and any adenine nucleotides released are insufficient to affect the kinetics of the enzyme, since the extent and rate with which a standard glutamate addition is

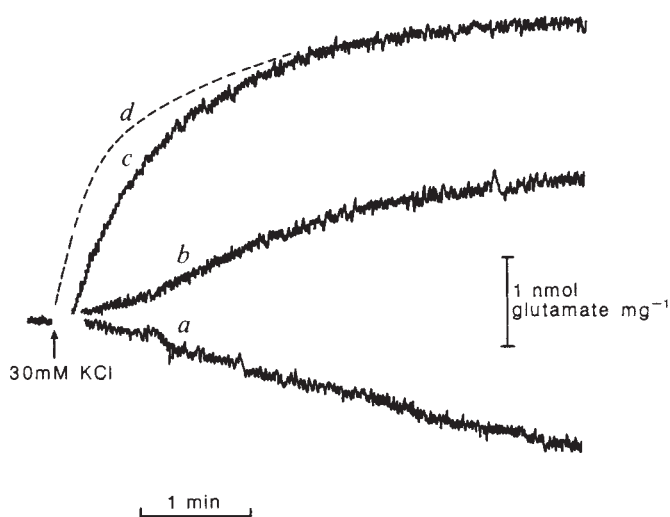


Fig. 1 Ca²⁺-dependent release of endogenous L-glutamate. *a*, Control (Ca²⁺ present); *b*, KCl added, EGTA present; *c*, KCl added, Ca²⁺ present; *d*, the rate at which a standard addition of 6.5 μ M L-glutamate was assayed was used to correct the Ca²⁺ + KCl trace for the hysteresis in the assay of the released amino acid.

Methods. Synaptosomes prepared from cerebral cortices of Dunkin-Hartley guinea pigs¹³ were suspended at 1.2 mg protein ml⁻¹ in 1.5 ml medium, (3.1 mM KCl, 122 mM NaCl, 1.2 mM MgSO₄, 0.4 mM KH₂PO₄, 5 mM NaHCO₃, 20 mM Na-Tes, 10 mM D-glucose, 16 μ M albumin, 1 mM NAD⁺, 12.5% (w/v) Ficoll, pH 7.4 at 30 °C in a cuvette within an Eppendorf filter photometer with fluorescence attachment (excitation 313 + 366 nm; secondary 430–3000 nm). 1.3 mM CaCl₂ or 0.5 mM EGTA were added at 2 min, followed at 5 min by the addition of 50 units of L-glutamate dehydrogenase (EC 1.4.1.3). When the initial exogenous glutamate was assayed, the recorder trace was started and 30 mM KCl added where indicated.

assayed is the same in the presence and absence of synaptosomes.

In contrast to the results obtained with the fluorometric assay, the KCl-induced release of label from synaptosomes that had accumulated ¹⁴C-L-glutamate for 20 min shows only slight dependency upon external Ca²⁺ (Fig. 2). Using HPLC, we determined that more than 90% of the isotope in the synaptosomes depolarized by adding KCl was present as glutamate plus aspartate. The discrepancy between the isotopic and fluorometric results indicate that there are two separate pools of glutamate, one labelled by ¹⁴C-L-glutamate, which does not require Ca²⁺ for its release, and the other, inaccessible to labelled glutamate within the timescale of this experiment, which is Ca²⁺-dependent. Because exogenous glutamate readily exchanges across the synaptosomal plasma membrane⁹, we assume that the Ca²⁺-dependent release detected by the fluorometric assay occurs from a noncytoplasmic compartment that does not equilibrate significantly with exogenous labelled glutamate under the conditions of the experiment.

The entry of ⁴⁵Ca and the elevation of cytosolic free Ca²⁺ show a sigmoidal dependency upon the extent of synaptosomal plasma membrane depolarization^{10,11}. Figure 3 shows that the rate of glutamate release upon depolarization with KCl in Ca²⁺-containing media is also sigmoidal, with a threshold potential of about 40 mV. In contrast, the Ca²⁺-independent release of glutamate increases linearly with the extent of depolarization, consistent with release from the cytoplasm by reversal of the electrogenic glutamate transporter¹².

D-Aspartate exchanges across the synaptosomal plasma membrane on the same carrier as L-glutamate⁹ and may therefore be used to exchange glutamate out of the cytoplasm. Fig. 4 shows that D-aspartate causes a Ca²⁺-independent release of

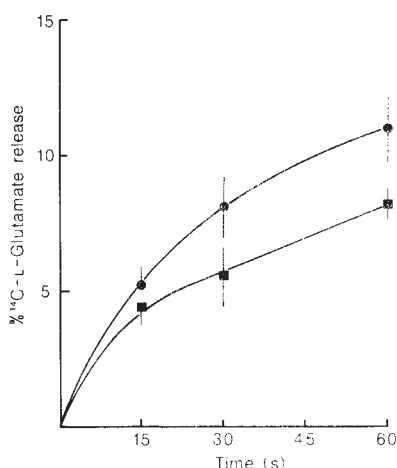


Fig. 2 Release of accumulated ^{14}C -L-glutamate is largely Ca^{2+} -independent. Synaptosomes were incubated at $1.5 \text{ mg protein ml}^{-1}$ in the initial medium described in Fig. 1, with the omission of Ficoll and NAD^+ . 1.3 mM CaCl_2 , ^3H -sucrose ($0.5 \text{ } \mu\text{Ci ml}^{-1}$) and $0.35 \text{ } \mu\text{M } ^{14}\text{C}$ -L-glutamate ($0.1 \text{ } \mu\text{Ci ml}^{-1}$) were added at 5 min. In one set of experiments 2 mM EGTA was added at 24 min to chelate exogenous Ca^{2+} . 20 mM KCl was added at 25 min, $250 \text{ } \mu\text{l}$ aliquots were centrifuged after a further 15s, 30s or 60s, and the retained ^{14}C quantified¹⁴. ●, 1.3 mM Ca present; ■, Ca chelated by 2 mM EGTA .

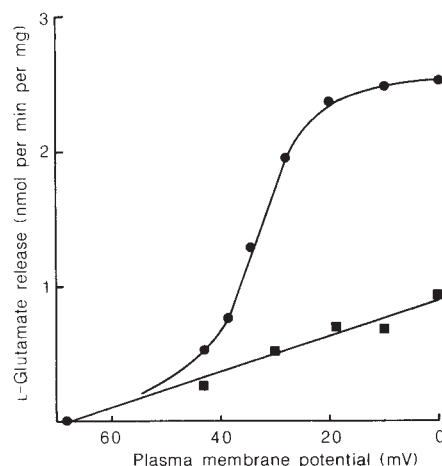
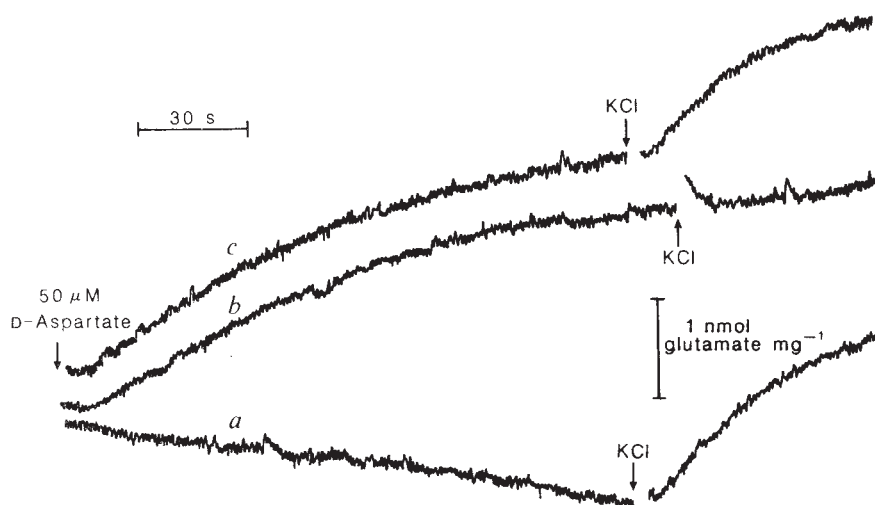


Fig. 3 Release of L-glutamate and plasma membrane depolarization. Synaptosomes were incubated as described in Fig. 1, except that the concentration of KCl added to initiate glutamate efflux varied from 6 to 48 mM . The plasma membrane potential was estimated from the ^{86}Rb distribution across the plasma membrane¹⁵. ●, 1.3 mM CaCl_2 present; ■, 0.5 mM EGTA present.

Fig. 4 Depletion of cytoplasmic L-glutamate by D-aspartate. *a*, control (1.3 mM CaCl_2 present, no D-aspartate); *b*, 0.5 mM EGTA present, D-aspartate added; *c*, 1.3 mM CaCl_2 present, D-aspartate added. Synaptosomes were incubated as described in Fig. 1. $50 \text{ } \mu\text{M}$ D-aspartate was added to initiate release of cytoplasmic glutamate. Where indicated 30 mM KCl was added. Note that D-aspartate pretreatment does not inhibit the subsequent Ca^{2+} -dependent release of glutamate by KCl .



the amino acid which is as extensive as that induced by KCl (Fig. 1). If the D-aspartate were exchanging glutamate out of the "transmitter" pool, defined functionally as the Ca^{2+} -dependent component in Fig. 1, one would predict a very substantial diminution of the Ca^{2+} -dependent efflux induced by the subsequent addition of 30 mM KCl . This however is not seen; thus D-aspartate, although exchanging extensively with cytoplasmic glutamate, does not deplete glutamate from the "transmitter pool" within 3 min, providing further evidence for a noncytoplasmic origin for the latter.

The total glutamate in the system (determined with Triton X100) does not increase when D-aspartate is employed to exchange glutamate out of the cytosol. Thus there is no resynthesis of the cytosolic pool within the time course of the experiment in Fig. 4. When D-aspartate and KCl are added simultaneously, the rate of glutamate efflux is equal to the sum of the rates induced by each agent separately. Thus $50 \text{ } \mu\text{M}$ D-aspartate released $0.52 \text{ nmol glutamate per min per mg protein}$; 30 mM KCl released $1.05 \text{ nmol per min per mg}$; while the two agents together released $1.58 \text{ nmol per min per mg}$.

These results show that 15–20% of the total glutamate in a preparation of guinea pig cerebrocortical synaptosomes can be

rapidly released in a Ca^{2+} -dependent manner from a noncytoplasmic pool by depolarization of the plasma membrane. The continuous enzymatic assay of released glutamate can provide within 5 min the complete kinetics for the release of glutamate from either the cytoplasmic or "transmitter" pool and should prove a powerful tool in the elucidation of the energetic and ionic requirements for the exocytosis of this putative transmitter.

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Phosphorylation of the nicotinic acetylcholine receptor regulates its rate of desensitization

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Recent studies have provided evidence for a role of protein phosphorylation in the regulation of the function of various potassium and calcium channels (for reviews, see refs 1, 2). As these ion channels have not yet been isolated and characterized, it has not been possible to determine whether phosphorylation of the ion channels themselves alters their properties or whether some indirect mechanism is involved. In contrast, the nicotinic acetylcholine receptor, a neurotransmitter-dependent ion channel, has been extensively characterized biochemically³ and has been shown to be directly phosphorylated^{4,5}. The phosphorylation of this receptor is catalysed by at least three different protein kinases (cyclic AMP-dependent protein kinase, protein kinase C and a tyrosine-specific protein kinase) on seven different phosphorylation sites⁶⁻⁸. However, the functional significance of phosphorylation of the receptor has been unclear. We have now examined the functional effects of phosphorylation of the nicotinic acetylcholine receptor by cAMP-dependent protein kinase. We investigated the ion transport properties of the purified and reconstituted acetylcholine receptor before and after phosphorylation. We report here that phosphorylation of the nicotinic acetylcholine receptor on the γ - and δ -subunits by cAMP-dependent protein kinase increases the rate of the rapid desensitization of the receptor, a process by which the receptor is inactivated in the presence of acetylcholine (ACh). These results provide the first direct evidence that phosphorylation of an ion channel protein modulates its function and suggest that phosphorylation of postsynaptic receptors in general may play an important role in synaptic plasticity.

The purified nicotinic acetylcholine receptor is a pentameric complex, which consists of four types of subunits in the stoichiometry $\alpha_2\beta\gamma\delta$ (ref. 3). This pentameric complex is fully functional biologically when reconstituted into phospholipid vesicles³. The ion transport properties of the reconstituted receptor can be analysed quantitatively by measuring the ACh-dependent transport of cations into vesicles by rapid kinetic techniques⁹. The nicotinic acetylcholine receptor in *Torpedo californica* postsynaptic membrane preparations was phosphorylated using the catalytic subunit of cAMP-dependent protein kinase in the presence of ATP (see Fig. 1 legend). This kinase phosphorylates a single serine residue in the γ -subunit and δ -subunit of the receptor that we have previously proposed to be serine 354 (γ -subunit) and serine 361 (δ -subunit)⁶⁻⁸. Under the standard conditions used, the γ - and δ -subunits were each phosphorylated to a final stoichiometry of 0.6–0.7 mol phosphate per mol of subunit (data not shown). Following the phosphorylation reaction, the postsynaptic membranes were solubilized with 1% Na-cholate and the nicotinic acetylcholine receptor purified by affinity chromatography¹⁰. Control non-phosphorylated receptor was prepared under identical conditions except that the catalytic subunit of cAMP-dependent protein kinase or ATP or both were omitted from the reaction mixture. (The three types of control preparations gave virtually identical results.) The purified receptor preparations were then reconstituted into phospholipid vesicles by cholate dialysis¹⁰, and quench-flow¹¹ and

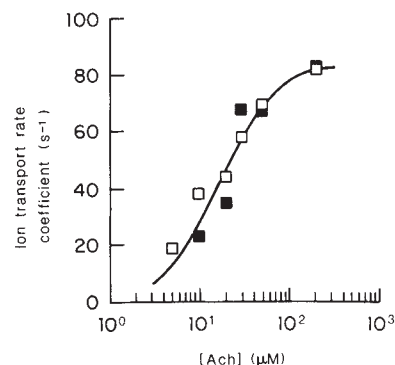


Fig. 1 Rate coefficient of ion transport (J_A) of the phosphorylated (0.6 mol phosphate per mol subunit) (■) and non-phosphorylated (□) receptor preparations as a function of acetylcholine (ACh) concentration.

Methods. Postsynaptic membrane preparations highly enriched in the nicotinic acetylcholine receptor were isolated from the electric organ of *T. californica*¹⁰ and alkali-treated²⁵ to increase the permeability of the membrane vesicles to exogenous reagents. The postsynaptic membranes were then diluted to 1.4 mg ml⁻¹ in the phosphorylation reaction mixture, which contained 20 mM Tris-HCl (pH 7.4), 2 mM EGTA, 1 mM EDTA, 10–20 mM MgCl₂, 1 mM β -mercaptoethanol, 1 mM ouabain, 1 mM Na₂VO₄, 20 μ g ml⁻¹ leupeptin, 20 μ g ml⁻¹ antipain, 20 units ml⁻¹ trypsin, 0.5 mM ATP and 100 nM of purified catalytic subunit of cAMP-dependent protein kinase. The phosphorylation reaction was carried out for 60 min at 30 °C. Na-cholate was then added to a final concentration of 0.3% and the mixture incubated for an additional 15 min at 30 °C. After cooling on ice, the receptor was solubilized with a final concentration of 1% Na-cholate and purified from the cholate extract on an ACh affinity column. The control non-phosphorylated receptor was prepared under identical conditions, except that ATP or the catalytic subunit, or both, were omitted from the reaction mixture. The two receptor preparations (~5 mg each) were reconstituted into phospholipid vesicles (asolectin) at a final concentration of ~0.45 mg ml⁻¹ receptor and 25 mg ml⁻¹ asolectin by cholate dialysis¹⁰, in the following buffer: 20 mM Tris Cl (pH 7.4), 100 mM NaCl, 50 mM KCl, 1 mM EDTA, 1 mM EGTA. The vesicles were frozen in liquid nitrogen in the absence (for quench-flow) or presence (stop-flow) of 10 mM 1,5-anthracene disulphonate and thawed before use. The ion transport properties of the reconstituted receptor preparations were analysed at 4 °C by measuring either the direct transport of ⁸⁶Rb⁺ (quench flow), or the quenching of the fluorescent dye, 1,5-anthracene disulphonate by Cs⁺ (stop-flow) as described previously^{11,12}. The rate coefficient for ion transport at ACh concentrations smaller than 50 μ M was obtained from a nonlinear least-squares fit of Cs⁺ influx curves, measured with the stop-flow technique and normalized to the values of J_A at 50 μ M or 200 μ M ACh which were obtained with the quench-flow technique as described elsewhere^{13,14}.

stop-flow¹² rapid kinetic techniques were used to analyse the properties of the ACh-dependent ion transport of the non-phosphorylated and phosphorylated receptor preparations.

We first determined the initial rates of ACh-dependent ion transport by the non-phosphorylated and phosphorylated acetylcholine receptor over a wide range of ACh concentrations (Fig. 1). The rates of ion transport characterized by the rate coefficient J_A of the non-phosphorylated and phosphorylated receptor had the same dependence on ACh concentration. This indicated that the dissociation constant of ACh for the sites that activate the receptor was unchanged by phosphorylation.

In contrast, when the rates of the rapid desensitization of the non-phosphorylated and phosphorylated receptor were measured directly, a striking difference was observed (Fig. 2). The rapid desensitization was measured by the quench-flow technique by preincubation of reconstituted vesicles with ACh for various periods of time (T) before the determination of the rate of ion transport^{13,14}. The data in Fig. 2 show the per cent ion transport activity remaining after preincubation of the non-

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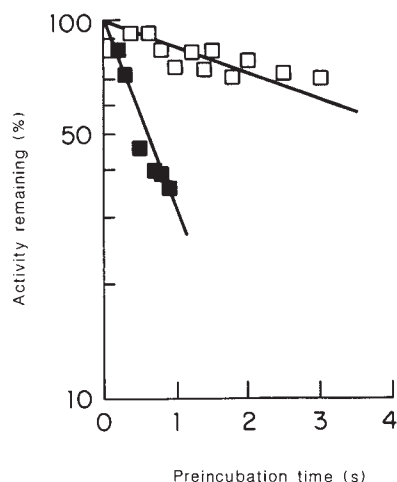


Fig. 2 Desensitization of the non-phosphorylated (□) and phosphorylated (■) nicotinic acetylcholine receptor. The reconstituted vesicles were preincubated with 10 μ M ACh for the times indicated and then the ion transport activity was measured for 12 ms with 50 μ M ACh using $^{86}\text{Rb}^+$ (refs 14, 26). The data were fitted to the following equation using a nonlinear least-squares program:

$$(J_A)_T = (J_A)_{T=0} e^{-\alpha T} \quad (1)$$

where $(J_A)_T$ is the ion transport rate coefficient after preincubation of the receptor with ACh for the period of time (T) given on the abscissa of the graph, $(J_A)_{T=0}$ is the ion transport rate coefficient prior to desensitization, and α is the desensitization rate coefficient. The 'activity remaining' given on the ordinate is $[(J_A)_T / (J_A)_{T=0}] \times 100$. □, Average data obtained with the three non-phosphorylated preparations ($\alpha = 0.15 \pm 0.02 \text{ s}^{-1}$); ■, averaged data obtained with two preparations of receptors phosphorylated to a stoichiometry of 0.6 mol phosphate per mol γ - and δ -subunits ($\alpha = 1.1 \pm 0.1 \text{ s}^{-1}$).

phosphorylated and phosphorylated receptor preparations with 10 μ M acetylcholine for the indicated times. The ion transport activity of both receptor preparations decreased as the preincubation time was increased and was described by a first-order rate law with the slope of the line proportional to the desensitization rate coefficient (α). The rate of desensitization of the non-phosphorylated receptor was similar to the rate previously described for the rapid desensitization in *Torpedo*¹⁴. The rate of desensitization of the phosphorylated receptor was 7–8 times faster than the rate of desensitization of the non-phosphorylated receptor. Figure 2 shows only the initial phase of the desensitization reaction to help visualize the difference in desensitization rates between the two preparations.

To investigate the effect of the stoichiometry of phosphorylation of the receptor on the desensitization process, we analysed the desensitization of receptor preparations phosphorylated to different extents. The data in Fig. 3a and b compare results obtained with receptor preparations that were phosphorylated on the γ - and δ -subunits to a stoichiometry of 0.4 mol phosphate per mol subunit and 0.6 mol phosphate per mol subunit respectively. With each of the preparations, when the time course of desensitization was followed to near completion, the data could not be fit with a single exponential and were best fit to the sum of two exponentials (see Fig. 3 legend). In contrast, control non-phosphorylated preparations displayed only a single exponential decay (data not shown). These results indicate that two different receptor populations existed in both phosphorylated preparations.

From the experiment shown in Fig. 2, we can equate the initial steeper slope seen in Fig. 3 with desensitization of the phosphorylated receptor and the final more shallow slope with that of the non-phosphorylated receptor. The intercept of the final slope with the ordinate represents the per cent of the ion transport associated with the receptors characterized by the slower

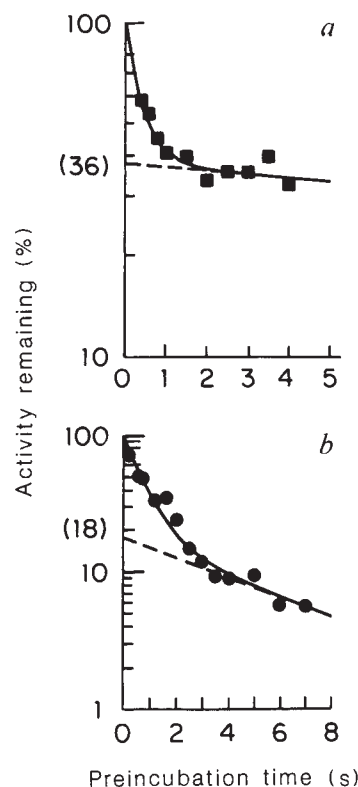


Fig. 3 Double exponential desensitization rate of the phosphorylated acetylcholine receptor. Desensitization of phosphorylated acetylcholine receptor preparations was measured as described in Fig. 2 legend. *a*, Receptor phosphorylated on the γ - and δ -subunits to a stoichiometry of 0.4 mol phosphate per mol subunit. 10 μ M ACh was used in the preincubation and 50 μ M ACh in the 12-ms influx measurement. *b*, Receptor phosphorylated on the γ - and δ -subunits to a stoichiometry of 0.6 mol phosphate per mol subunit. 50 μ M ACh was used in the preincubation and in the 12-ms influx measurement. The 'activity remaining' given on the ordinate is $[(J_A)_T / (J_A)_{T=0}] \times 100$. The rate coefficients of the two exponentials were calculated according to the following equation:

$$(J_A)_T = (J_A)_{T=0} [a e^{-\alpha_N T} + b e^{-\alpha_P T}]$$

$(J_A)_T$ and $(J_A)_{T=0}$ are defined in Fig. 2 legend and α_N and α_P indicate the constants characteristic of the non-phosphorylated and phosphorylated receptor, respectively; a and b represent the fractions of the preparation associated with the non-phosphorylated and phosphorylated receptors, respectively. All parameters (a , b , α_N , and α_P) were determined from a nonlinear least-squares fit of the data to the above equation.

desensitization rate. In the receptor preparation that was phosphorylated to a stoichiometry of 0.4 mol phosphate per mol subunit, 64% of the preparation desensitized with the increased rate while 36% desensitized with the slow rate (Fig. 3a). In the receptor preparation that was phosphorylated to a stoichiometry of 0.6 mol phosphate per mol subunit, 82% of the preparation desensitized with the increased rate while 18% desensitized with the slow rate (Fig. 3b).

Assuming that phosphorylation of the γ - and δ -subunits occurs independently, four different populations of receptors would exist: those that are phosphorylated on neither subunit, those that are phosphorylated on only the γ -subunit, those that are phosphorylated on only the δ -subunit, and those that are phosphorylated on both subunits. Given this assumption, and the stoichiometry of phosphorylation of the receptor preparations used for the experiments in Fig. 3, the percentage of receptor molecules that would contain at least one phosphate group would be 64% and 84%, respectively. These values are in excellent agreement with the 64% and 82% calculated (from the intercepts of the final slopes in Fig. 3 with the ordinate) for

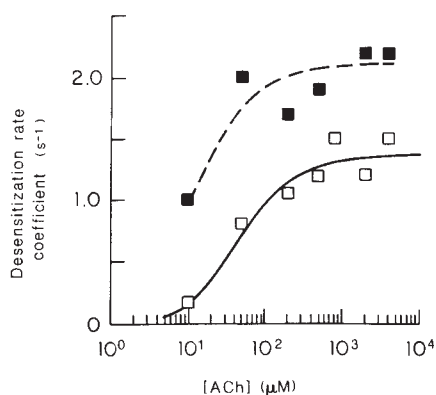


Fig. 4 Rate coefficient for desensitization of the phosphorylated receptor (α_P , ■) and non-phosphorylated receptor (α_N , □) as a function of acetylcholine concentration. Receptor preparations were phosphorylated to a final stoichiometry of ~ 0.6 mol phosphate per mol γ - and δ -subunits. Desensitization of the nonphosphorylated and phosphorylated receptor was measured at various ACh concentrations. The rate coefficient of the phosphorylated receptor was obtained by analysis of the desensitization data according to the equation given in Fig. 3 legend using the values of α_N determined experimentally and the values of a and b observed in Fig. 3b. Four different phosphorylated and non-phosphorylated preparations were used to obtain the α values over the concentration range shown.

the percentage of receptors that desensitize with the fast rate. The simplest model which accounts for these results is that the ion transport rate is unchanged by phosphorylation (see Fig. 1) and that phosphorylation of either the γ - or δ -subunit or both results in the observed increase in the rate of desensitization.

The dependence on the ACh concentration of the desensitization rate coefficient (α) of the non-phosphorylated and phosphorylated receptor preparations is shown in Fig. 4. The rate of desensitization of the phosphorylated receptor was significantly greater than that of the dephosphorylated receptor over the whole concentration range. The most dramatic differences were seen at low concentrations of ACh, since the ACh concentration required to reach the half-maximal desensitization rate was several-fold lower for the phosphorylated receptor.

The results presented here demonstrate that phosphorylation of the γ - and δ -subunits of the nicotinic acetylcholine receptor increases the rate of the rapid desensitization of the receptor. Our results also indicate that phosphorylation of the receptor does not affect either the rate of ion transport or the apparent dissociation constant of acetylcholine for the activation of ion transport. In addition, preliminary results from patch-clamp studies of reconstituted vesicles¹⁵ indicated that phosphorylation of the γ - and δ -subunits has little or no effect on the single-channel conductance or mean channel open-time (D. W. Tank and R.L.H., unpublished results).

Recent studies with intact muscle preparations have shown that forskolin, an activator of adenylate cyclase, dramatically increases the rate of desensitization of the nicotinic acetylcholine receptor^{16,17}. In addition, it has recently been reported that agents that activate protein kinase C increase the rate of desensitization of the acetylcholine receptor in cultured myotubes¹⁸. These *in vivo* results, together with the *in vitro* results of the present study, suggest that phosphorylation of the nicotinic acetylcholine receptor by either cAMP-dependent protein kinase or protein kinase C increases its rate of desensitization. This is interesting in the light of our proposal⁸ that the phosphorylation sites for cAMP-dependent protein kinase and for protein kinase C are located on a common region of each subunit, with the two phosphorylation sites on the δ -subunit being within 16 amino acids of each other. These regions are the major intracellular loops adjacent to the membrane-spanning α -helices that are proposed to form the ion channel in the

theoretical models of the receptor¹⁹⁻²³. Our results suggest that these intracellular loops are intimately involved in the process of desensitization. Desensitization has been proposed to be a form of short-term regulation, in the second to minute time range, of synaptic efficacy (see, for example, refs 3, 9, 24). Although desensitization of the nicotinic acetylcholine receptor clearly occurs in non-phosphorylated receptor preparations, it is also clear that phosphorylation can dramatically modify the desensitization process. In addition, the present results support the idea (see, for example, ref. 3) that phosphorylation of postsynaptic receptors is a plausible although speculative molecular model for short-term and long-term learning at the neuronal level.

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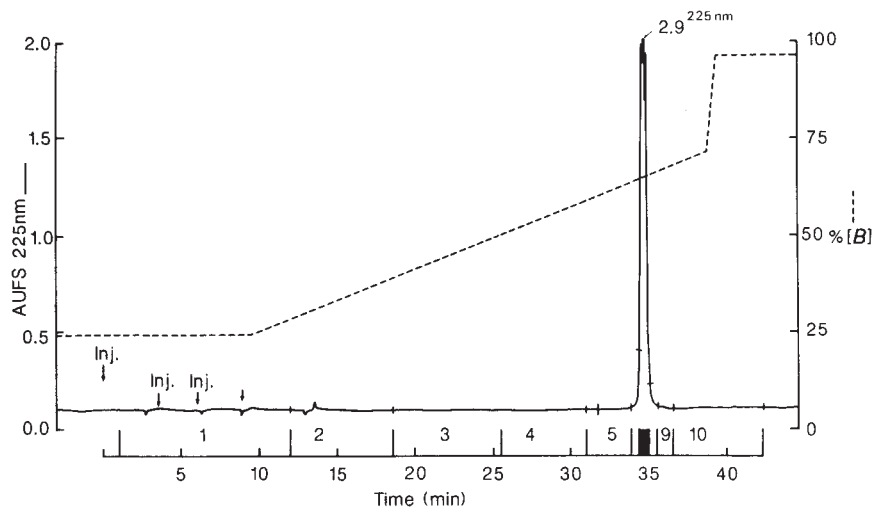
Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid

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A variety of hypophysiotropic peptides or proteins have been reported to be present in mammalian gonads. Inhibin, a hormone that under most circumstances selectively suppresses the secretion of follicle-stimulating hormone (FSH) but not luteinizing hormone (LH), has been isolated from the gonadal fluids of several species¹⁻⁵ and characterized as a heterodimeric protein consisting of α - and β -polypeptides associated by disulphide bonds. The complete amino-acid sequences of the precursors of porcine^{6,7} and human⁷ inhibin α -subunits and two distinct porcine inhibin β -subunits

Fig. 1 Elution profile on analytical HPLC of FRP bioassayed and purified as described below. AUFS, absorbance units full scale. **Bioassay:** Rat anterior pituitary glands were dissociated enzymatically and the cells plated as described previously^{3,15}. After washing the cells twice, treatments were added and allowed to remain on the cells for 48–72 h, after which fluids were removed and tested for FSH using a radioimmunoassay kit provided by the National Hormone and Pituitary Program of NIADDK. For reverse-phase HPLC columns, aliquots for assays (0.1–1.0% of total fraction volume but never less than 5 μ l) were measured with micropipettes and plastic tips and were transferred into polypropylene tubes containing bovine serum albumin (10 μ l of 10 mg ml⁻¹) and dried in a Savant rotary evaporator. For gel permeation and ion-exchange columns where desalting was necessary before assay, small aliquots were removed and transferred to glass tubes containing 0.5 ml of 10 mM HEPES+0.1% bovine serum albumin pH 7.5. Squares of dialysis tubing with a M_r cutoff of ~1,000 were secured over the tops with rubber bands. The tubes were inverted and dialysed against 10 mM HEPES, pH 7.5. The retentates were then dried using a Savant rotary evaporator. All fractions were resuspended in cell culture assay medium. **Ammonium sulphate precipitation and ultrafiltration:** Porcine follicular fluid (6,050 ml) was made available to us by the Contraceptive Development Branch of NICHD, and thawed and centrifuged at 700g for 5 min to separate cell debris. An equal volume of Schwartz/Mann ultrapure 100% saturated ammonium sulphate, adjusted to pH 7.8 with ammonium hydroxide, was added dropwise to the crude supernatant over 2–3 h. The mixture was centrifuged at 8,200g for 30 min and the supernatant and precipitate were separated. The supernatant was ultrafiltered and concentrated using a Millipore Pellicon Cassette system with 5 square feet of filters of 10,000 M_r cutoff at a rate of 300 ml min⁻¹, using a peristaltic pump with back pressure applied until the filtration rate was 20 ml min⁻¹. The retentate was washed several times with 10 mM HEPES and 0.05% dimethyl sulphide, pH 7.5. The final retentate was collected (6,050 ml-equiv. in 2.24 l). **Preparative reverse-phase HPLC:** 500 ml-equiv. or less per run was processed on a Waters Prep 500A using Vydac end-capped C₈ silicas, 15–20 μ m particle size in a 5 $\times$ 30 cm cartridge. The column temperature was thermostated at 60°C. Buffer A was 0.1% H₃PO₄, 0.28% triethyl ammonium (TEA), pH 6.5; buffer B was 60% *n*-propanol in buffer A. Columns were loaded at 20% buffer B; gradient used was 20% B to 65% B in 45 min. Flow rate was 75 ml min⁻¹. FSH releasing activity eluted between retention volumes 825 and 1,360 ml from the start of the gradient. **Gel permeation chromatography:** Active zones from preparative reverse-phase HPLC were pooled, lyophilized and brought up in gel permeation column eluant. Column eluant was 6 M guanidine-HCl, 0.1 M ammonium acetate, 0.05% dimethyl sulphide, pH 4.75. Eluant was filtered at 0.22 μ m and degassed before use. A 10 $\times$ 120 cm column (volume 8,600 ml) of Sepharose CL-6B was used at a flow rate of 180 ml h⁻¹. FSH releasing activity eluted between K_{AV} 0.54 and 0.71. **Cation exchange on FPLC:** Active fractions from the gel permeation chromatography were pooled and dialysed against Milli H₂O+0.01% triethyl sulphide using tubing with a M_r cutoff of 1,000. The retentate was lyophilized and brought up in buffer A. Buffer A was 50 mM sodium formate, 4 M urea, 1 mM CHAPS (3-[(cholamidopropyl)dimethylammonio]-1-propanesulphonate) in Milli Q H₂O, pH 4.3. Buffer B was 1 M NaCl in buffer A. Buffers were filtered at 0.22 μ m and degassed before use. FRP fractions were processed in three equal batches using a Pharmacia FPLC system equipped with a Mono S HR 16/10 column, V_t = 20 ml. Flow rate, 8 ml min⁻¹. The sample was loaded in buffer A and the column was then run isocratically at 15% buffer B for 7 min followed by a gradient from 15% to 50% B in 15 min. Two active zones of FRP resulted from cation exchange. The later-eluting zone, which eluted from 0.43 to 0.60 NaCl, was purified further. **Semi-prep reverse-phase HPLC:** FRP was purified further and desalted on a Beckman HPLC using Vydac 1 $\times$ 30 cm columns packed with end-capped C₈ silicas, 5 μ m particle size. Buffer A was 0.1% trifluoroacetic acid (TFA); buffer B was 80% CH₃CN in 0.1% TFA. Columns were thermostated at 40°C and run at 2.5 ml min⁻¹ isocratically at 25% buffer B for 17 min followed by a gradient to 55% B in 30 min. Activity eluted between retention volumes 60 and 65 ml from the start of the gradient. **FPLC gel permeation:** Active zones from analytical reverse-phase HPLC of FRP were processed on a Pharmacia FPLC system using tandem Superose 12B columns, 10 μ m, 10 $\times$ 300 mm each. Column eluant was 6 M guanidine-HCl, 0.1 M ammonium acetate, 0.05% dimethyl sulphide, pH 4.75 in Milli Q H₂O. Buffer was filtered at 0.22 μ m and degassed before use. Flow rate, 0.4 ml min⁻¹. Activity was eluted between K_{AV} 0.26 and 0.31. **Analytical reverse-phase HPLC:** The active zone of FRP was processed using a Vydac C₈, 0.46 $\times$ 25 cm column using the Beckman system at 40°C. Buffer A was 0.1% TFA, buffer B was 80% CH₃CN in 0.1% TFA. Approximately 1,000 ml-equiv. was loaded at a time, starting initially at 25% buffer B, 1.2 ml min⁻¹, and running a gradient to 45% B in 30 min with a flow rate of 0.7 ml min⁻¹. The active fractions of FRP were pooled, then concentrated on a steep gradient of 25% B initially to 72% B in 30 min. FSH releasing activity eluted as shown.



(β_A and β_B)⁶ have been deduced from complementary DNA sequences. Gonadotropin releasing peptides have also been found in the gonad and have generally been shown to be active in radioreceptor assays for gonadotropin releasing hormone (GnRH) but to exhibit different chromatographic and immunological characteristics from those of GnRH^{6–14}. During our purification of inhibin from porcine follicular fluid, we noted fractions that could stimulate the secretion of FSH by cultured anterior pituitary cells³. We report here the purification of an FSH releasing protein (FRP) and its characterization by SDS-polyacrylamide gel electrophoresis under non-reducing and reducing conditions and by partial sequence analysis. Our results indicate that porcine gonadal FRP is a homodimer consisting of two inhibin β_A -chains linked by disulphide bonds. FRP is highly potent (50% effective concentration (EC_{50}) ~ 25 pM) in stimulating the secretion and biosynthesis of FSH but not of LH or any other pituitary hormone. In contrast to the effects of GnRH and other reported gonadal gonadotropin releasing fractions, the action of FRP is not mediated by GnRH receptors.

The dialysed supernatant from a 50% ammonium sulphate precipitation of 6 litres of porcine follicular fluid was subjected to preparative HPLC as described previously³. FSH releasing activity was observed in the early-eluting, retarded fractions, and was thereby separated from the two major FSH release inhibiting (inhibin) zones, which were either not retarded or eluted later.

We hypothesized that an FSH releasing activity was due to an FSH releasing protein and we decided to purify this (Table 1) and characterize it chemically. The assay for FRP was based on the ability of the protein to stimulate basal FSH secretion by cultured rat anterior pituitary cells^{3,15}. The FSH releasing zones from preparative reverse-phase HPLC were subjected to gel filtration on Sepharose CL6B in a strongly dissociating buffer containing 6 M guanidine-HCl. We noted at this point that FRP behaved similarly on gel permeation to the 32,000 (32K)-relative molecular mass (M_r) form of inhibin which had been separated earlier by preparative HPLC. The FSH releasing fractions were then purified further by cation exchange chromatography on fast protein liquid chromatography using Mono-S in a buffer containing urea and the detergent CHAPS at pH 4.3, and eluted by a gradient of 0–1 M NaCl. Two regions of FSH releasing activity were detected. The later-eluting zone had the higher specific activity and was purified further by semi-preparative reverse-phase HPLC, gel permeation on FPLC in 6 M guanidine-HCl and two steps of analytical reverse-phase HPLC (Fig. 1). On SDS-polyacrylamide gel electrophoresis (Fig. 2), purified non-reduced FRP showed a major band of M_r ~ 28,000. Bioassay of fractions that eluted from a similar gel confirmed that FSH releasing activity was associated with this band. Under reducing conditions, FRP converted to a species of M_r ~ 15,000. An aliquot of highly purified FRP was subjected to Edman degradation and the N-terminal sequence (Fig. 3) was found to

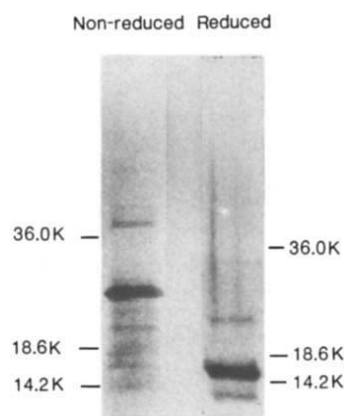


Fig. 2 SDS-polyacrylamide electrophoresis of FRP. Aliquots (0.3–0.8 μ g) of the FRP fraction used for Edman degradation (see Fig. 3) were treated with 2% SDS in the presence and absence of 5% (v/v) β -mercaptoethanol for 15 min at room temperature and subjected in separate experiments to electrophoresis in polyacrylamide gels consisting of a stacking gel (5% acrylamide) and a gel of decreasing pore size (10–20% acrylamide). Bands were detected by silver staining. The mobilities of protein markers (glyceraldehyde 3-phosphate dehydrogenase (36K), β -lactoglobulin (18.6K) and α -lactalbumin (14.2K)) are indicated.

be identical to the N-terminal 38 residues of the porcine inhibin β_A -chain⁶. Another aliquot was reduced, *S*-carboxymethylated and purified by HPLC. The major protein zone was digested with clostripain and the fragments were purified by HPLC. The sequences of three such fragments were determined by Edman degradation to be identical to residues 70–85, 88–102 and 103–116 of porcine inhibin β_A (Fig. 3). These results are consistent with the hypothesis that porcine gonadal FRP is a homodimer consisting of two inhibin β_A -chains linked by disulphide bonds.

Both of the β -subunits of porcine inhibin, β_A and β_B , possess significant homology with human transforming growth factor- β (TGF- β)¹⁶ and are considered to be members of the same gene family⁶. It is noteworthy that TGF- β , like FRP, exists as a homodimer linked by disulphide bonds. The presence of the inhibin β_A dimer (FRP) raises the possibility of the existence of an inhibin β_B dimer as well as perhaps inhibin β_A - β_B heterodimers.

Highly purified FRP stimulates FSH secretion by cultured anterior pituitary cells in a dose-related fashion, exhibiting an EC_{50} value of ~ 25 pM (Fig. 4a). FRP does not stimulate the secretion of LH (Fig. 4b), prolactin, growth hormone or adrenocorticotrophic hormone, and in fact suppresses the basal and stimulated release of the latter two hormones (data not shown). FRP can be distinguished from GnRH in several ways. FRP, which is a potent releaser of FSH, has no effect on the

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1      36      50
GLECDGKVNICKCKOFFVSFKDIGWNDWIAPSGYHANYCEGCPSHIAG-
70      85      88
TSGSSLSFHSSTVINHYRMGRGHSFPANLKSCCVPTKLRPMSMLYYDDGQNI-
102 103      116
IKKQIONMIVEECGCS

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Fig. 3 Sequence analysis of FRP and structural comparison of FRP and the β_A -subunit of porcine inhibin. Identified residues of FRP are indicated by underlining of the porcine β_A -subunit structure⁶. Eighty-three amino acids of the sequence of porcine FRP were established and all were homologous with the corresponding 83 out of 116 residues of porcine inhibin β_A . FRP from the final reverse-phase HPLC was subjected to Edman degradation on an Applied Biosystems 470A gas-phase protein sequencer. Phenylthiohydantoin (PTH) amino-acid analysis was performed by reverse-phase HPLC with a Hewlett-Packard 1084B liquid chromatograph. Repetitive yields of stable PTH-amino acids were >94%. Relative PTH-amino-acid yields of consecutive Edman cycles agreed with corresponding values found by Edman degradation of synthetic peptides. Details of the method of sequence analysis are described elsewhere²². The amino-acid sequence of residues 1–38 of FRP was established by analysis of ~ 0.9 nmol of unmodified peptide. On the basis of the PTH-amino-acid analysis, the identified peptide represented >90% of the peptide of the fraction analysed. The FRP fragments, FRP(70–85), FRP(88–102) and FRP(103–116), were analysed after *S*-carboxymethylation²³ of 1.2 nmol of FRP, reverse-phase HPLC of the carboxymethylated FRP, its digestion with clostripain²⁴ (Substrate enzyme weight ratio, 20:1; 3.5 h at 37 °C; buffer, 0.05 M Quadrol, 5 mM $CaCl_2$, 1 mM dithiothreitol, 6% (v/v) *n*-propanol, pH 8.0) and purification of the fragments on reverse-phase HPLC (Vydac C_{18} column, 0.46 $\times$ 25 cm, pore size, 300 Å; elution: 0.05% TFA in aqueous acetonitrile; 1.0 ml min⁻¹, 31 °C). 100% of each purified fragment (corresponding to 200–400 pmol of peptide as estimated on the basis of PTH-amino-acid analysis) was subjected to Edman degradation.

secretion of LH whereas GnRH releases both gonadotropins (Fig. 4b). GnRH acts immediately to stimulate gonadotropin secretion; by contrast, the onset of action of FRP is delayed by more than 4 h, and is maximally effective only after 24 h. Pituitary cells treated with the highest concentrations of FRP for 72 h secrete 70–100% as much FSH during that period as do cells exposed to plateau levels of GnRH. On the other hand, FRP is considerably more potent than GnRH, typically exhibiting EC_{50} values of less than 1/25th that of GnRH (Fig. 4a). The administration of high concentrations of both FRP and GnRH together stimulates more FSH secretion than does the addition of maximal levels of either substance alone. GnRH receptor antagonists which completely prevent the release of FSH and LH due to GnRH *in vitro* have no effect on the secretion of FSH mediated by FRP. These results indicate that FRP acts independently of the GnRH receptor. Prolonged exposure of pituitary cells to GnRH results in depletion of cellular FSH stores whereas FRP, which releases similar amounts of FSH into the medium, actually increases the amounts of stored FSH (Table 2). FRP therefore appears to have a greater stimulatory effect on the biosynthesis of FSH than does GnRH, when delivered continuously.

Purified porcine inhibin³ lowers baseline production of FSH

Table 1 Purification of FSH releasing protein

Step	Activity elutes	μ g protein per ml-equiv.	Bioassay EC_{50} (ng protein per ml medium)
Starting material: 6,050 ml porcine follicular fluid		65,000	
1. 50% $(NH_4)_2SO_4$	Supernatant	10,000	
2. Preparative HPLC; Vydac C_4 ; TEA-phosphate, pH 6.5/propanol at 60 °C	Retarded	700	2,800
3. Gel filtration; Sepharose CL 6B; 6 M guanidine-HCl, 0.1 M NH_4CH_3COOH , pH 4.75	K_{av} = 0.54–0.71	130	730
4. Cation exchange FPLC; Mono S; 4 M urea, 1 mM CHAPS, 0.1 M $NaCOOH$, pH 4.3; 0–1 M NaCl	0.43–0.6 M NaCl	8	96
5. Semi-preparative HPLC; Vydac C_8 ; 0.1% TFA/ CH_3CN	Retarded	0.7	25
6. Gel permeation FPLC; Superpose 12B, 6 M guanidine-HCl, 0.1 M NH_4CH_3COOH , pH 4.75	K_{av} = 0.26–0.31	0.3	5
7. Analytical HPLC; Vydac C_8 ; 0.1% TFA/ CH_3CN			
8. Repeat step 7	Retarded	0.02	0.5–1

Ultrafiltration or dialysis following steps 1, 2 and 3. Details and explanation of abbreviations are given in Fig. 1 legend. Results of the bioassay are expressed as the concentration (ng protein per ml) required of a fraction to elicit a half-maximal response in the cell culture bioassay¹⁵.

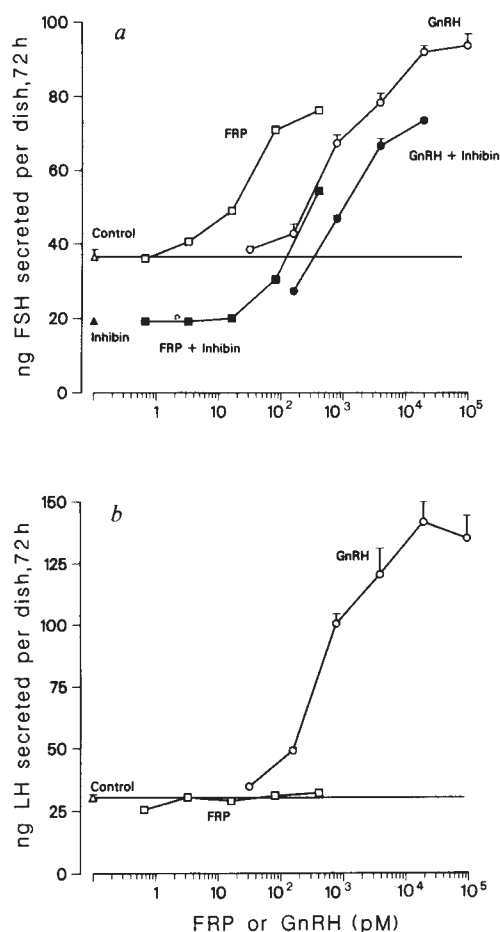


Fig. 4 *a*, Effects of FRP, porcine inhibin and GnRH on FSH secretion by rat anterior pituitary cells in primary culture prepared as described in Fig. 1 legend. All treatments were added simultaneously and incubation was continued for 72 h. Highly purified 32K porcine inhibin was given at 40 pM. *b*, Effects of GnRH and FRP on LH secretion by rat anterior pituitary cells in primary culture.

and will thereby obscure the effects of the lower concentrations of FRP (Fig. 4*a*). The pharmacological interaction between the two proteins is functionally antagonistic although each has effects in the absence of the addition of the other. The existence of gonadal FRP may have been overlooked previously because of the presence of inhibin, whose 32K form is not separated from FRP by gel-permeation chromatographic methods.

The physiological significance and anatomical distribution of FRP remain to be investigated. Previous reports of an FSH releasing factor have assumed its presence in the hypothalamus and partial purification of a hypothalamic FSH releasing factor of smaller size and more rapid action than gonadal FRP has been claimed¹⁷⁻²¹. At this time, we do not know whether FRP is secreted *per se* or is generated by extracellular processing. The high potency of FRP on pituitary cells in modulating hormone secretion and biosynthesis suggests the presence of high-affinity FRP receptors and supports the regulatory potential of this protein. In view of its similarity of sequence and subunit organization to TGF- β , FRP may have a variety of autocrine

and paracrine actions within the gonad and perhaps other tissues.

With the information available, we can propose the existence of a novel and complex regulatory system involving differential subunit association giving rise to dimers with biologically opposite effects. It is clearly important to elucidate the conditions that determine whether inhibin β_A -subunits are combined with the dissimilar inhibin α -subunits to yield inhibin, which suppresses FSH production, or with each other, yielding FRP, a potent stimulator of FSH production.

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Pituitary FSH is released by a heterodimer of the β -subunits from the two forms of inhibin

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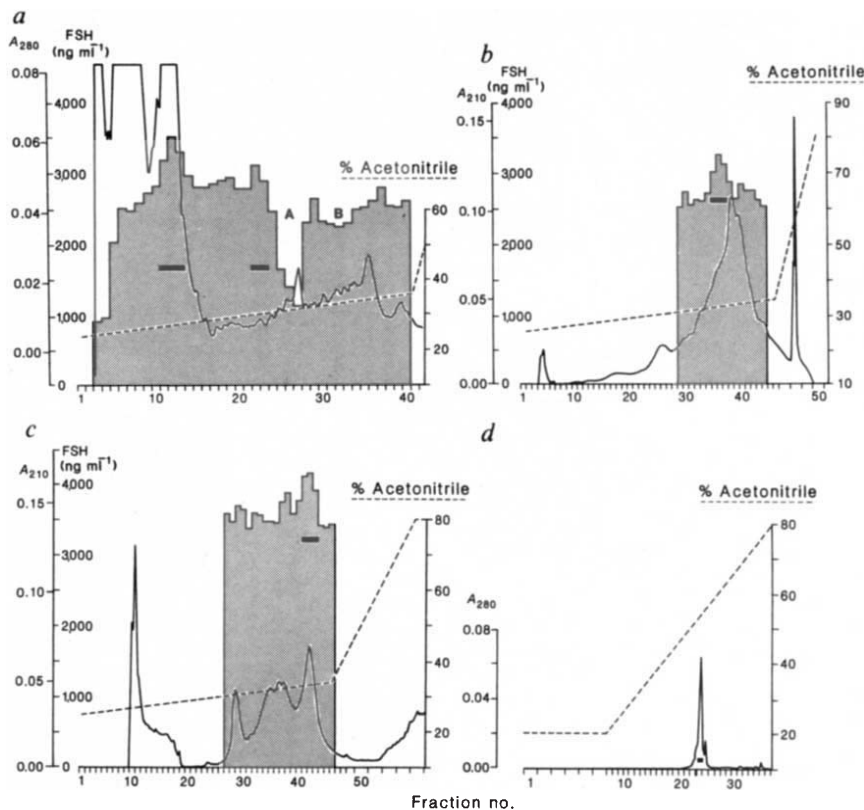
Inhibin is a gonadal protein that specifically inhibits the secretion of pituitary follicle-stimulating hormone (FSH). Two forms of inhibin (A and B) have been purified from porcine follicular fluid¹ and characterized as heterodimers of relative molecular mass (M_r) 32,000 (ref. 2). Each inhibin is comprised of an identical α -subunit of M_r 18,000 and a distinct but related β -subunit of M_r 13,800-14,700 linked by interchain disulphide bond(s). Throughout the purification of inhibins, we consistently observed two fractions which stimulated the secretion of pituitary FSH. We report here the isolation of one of the FSH-releasing proteins; it has a M_r of 24,000 and its N-terminal sequences up to residue 32 are

Table 2 Effects of GnRH or FRP on amounts of intracellular, secreted or total FSH in anterior pituitary cell cultures over a 48-h period

	ng FSH per well		Total
	Intracellular	Secreted	
Control	135.6 $\pm$ 1.9	79.7 $\pm$ 2.0	216.3 $\pm$ 0.2
100 nM GnRH	46.8 $\pm$ 2.1	161.0 $\pm$ 0.9	207.8 $\pm$ 2.9
0.2 nM FRP	176.4 $\pm$ 3.6	165.8 $\pm$ 6.4	342.2 $\pm$ 3.0

Fig. 1 Purification of the FSH-releasing substance, activin, from porcine follicular fluid. Sequential chromatography of the Sephacryl S-200 purified material was processed through: a Vydac C₄ semi-preparative column and eluted with acetonitrile in Et₃NP buffer (a); a Vydac C₄ semi-preparative column and eluted with acetonitrile in trifluoroacetic acid (TFA) solvent system (b); a Vydac phenyl semi-preparative column and eluted with acetonitrile in Et₃NP buffer (c); and an Aquapore RP-300 C₈ analytical column and eluted with acetonitrile in TFA solvent system (d). Solid bars, active material.

Methods. The FSH-releasing substance was purified by a procedure similar to that used in the isolation of inhibins A and B¹. Briefly, 500 ml of porcine follicular fluid was defrosted and the cell debris removed by centrifugation. The supernatant was diluted to 10 times its original volume with 0.1 M NaCl/10 mM Tris-HCl, pH 7, and pumped simultaneously onto eight heparin-Sepharose (Pharmacia) columns (3.5×9 cm) at 40 ml h⁻¹ per column¹. The adsorbed proteins were removed by washing with 1 M NaCl/10 mM Tris-HCl, pH 7. The protein-containing fractions were pooled and dialysed overnight (Spectrapor No. 3 membrane tubing, M_r cutoff 3,500, Spectrum Medical Industries) against 30% (v/v) acetic acid. The retentate was centrifuged to remove a white precipitate and the supernatant divided into eight equal portions for application to eight 5×100 cm columns of Sephacryl S-200 Superfine (Pharmacia) and developed with 30% acetic acid at 20 ml per 22 min as before¹. The fractions containing the 32,000-M_r inhibins and FSH-releasing activity, which eluted after inhibin, were pooled and lyophilized (see Fig. 1a in ref. 1). For reverse-phase HPLC, two solvent systems were used. In the Et₃NP system, solvent A consists of 0.25 M triethylammonium phosphate (pH 3), and solvent B is 80% (v/v) acetonitrile in solvent A. In the TFA solvent system, solvent A contains 1 ml TFA in 999 ml of water, and solvent B is 1 ml of TFA in 199 ml of water and 800 ml of acetonitrile. The lyophilized material (53 mg) was dissolved in 40 ml 0.2 M acetic acid and filtered through a Millex-HA 0.45 µm filter (Millipore). The filtrate was applied directly to a Vydac 5 µ C₄ column (10×250 mm). After all the filtrate had been loaded, the column was washed with the aqueous solvent A until the ultraviolet absorption reached baseline. The inhibin and FSH-releasing activities were separated with the indicated gradient of acetonitrile in Et₃NP solvent system on a Beckman 322 gradient liquid chromatography system equipped with a Spectroflow 747 UV detector (Kratos Analytical Instruments), a Soltec 220 recorder (Soltec) and a RediRac 2112 fraction collector (LKB) as shown in a. Two zones of inhibin activity, designated inhibins A and B, were separated, as were two zones of FSH-releasing activity denoted by the two solid bars. The FSH-releasing fractions denoted by the black bar and eluting at 30% acetonitrile were pooled and, after mixing with an equal volume of 0.2 M acetic acid, pumped directly onto a Vydac 5 µ C₄ column, 10×250 mm, and eluted with the indicated gradient of acetonitrile in TFA solvent system at 6 ml per 2 min as shown in b. The active material (denoted by the solid bar) was pooled, diluted to twice its original volume and chromatographed on a Vydac 5 µ phenyl column (10×250 mm) with the indicated gradient of acetonitrile in Et₃NP solvent system at 2 ml per 2 min (c). The active material (solid bar) from four chromatographic runs identical to those in c was pooled and, after dilution with 0.2 M acetic acid, concentrated by chromatography on an Aquapore 10 µ RP-300 column (4.6×250 mm) using the indicated gradient of acetonitrile in TFA system at 0.5 ml min⁻¹ as shown in d. From 2 l of follicular fluid, 32 µg of activin was isolated.



identical to those of each β -subunit of inhibins A and B. In the presence of reducing agents, SDS-polyacrylamide gel electrophoresis resolves the FSH-releasing substance into two subunits which are identical in their migration behaviour to the reduced β -subunits of inhibins A and B. Based on the N-terminal sequence data and M_r of the intact and reduced molecules, we propose that the FSH-releasing substance, which is active in picomolar concentrations, is a heterodimeric protein composed of the two β -subunits of inhibins A and B linked by interchain disulphide bond(s). The structural organization of the FSH-releasing substance is homologous to that of transforming growth factor- β (TGF- β)³, which also possesses FSH-releasing activity in the same bioassay⁴. We suggest that the substance be called activin to signify the fact that it has opposite biological effects to inhibin.

Frozen porcine follicular fluid (collected by J.R. Scientific and procured through the Contraceptive Development Branch, Center for Population Research, National Institute of Child Health and Human Development) was used as starting material for the purification of the FSH-releasing substance. After defrosting, the follicular fluid was diluted with buffer and purified through successive steps of heparin-Sepharose affinity chromatography, size fractionation on Sephacryl S-200 in acidic

medium and four reverse-phase HPLC steps as described in Fig. 1 legend. The inhibin and FSH-releasing activities were monitored by an *in vitro* bioassay using a monolayer culture of dissociated rat anterior pituitary cells¹. Inhibin-containing fractions were identified by their ability to inhibit the 48-h basal release of FSH but not that of luteinizing hormone or other pituitary hormones in comparison with control cultures; FSH-releasing fractions were identified by their ability to release FSH above basal level during the 48-h incubation in comparison with control cultures. Two forms of FSH-releasing substance, eluting at 27% and 30% acetonitrile, were noted in the first reverse-phase HPLC step (Fig. 1a). The later-eluting form was purified to homogeneity by three more HPLC steps (Fig. 1b-d). The purified protein yielded a single band of apparent M_r 24,000 on SDS-polyacrylamide gel electrophoretic (SDS-PAGE) analysis under non-reducing conditions (Fig. 2a).

The homogeneous 24,000-M_r material, now called activin, exhibited a half-maximal stimulation of FSH release at a concentration of 3.7 ± 0.5 ng ml⁻¹ ($\sim 1.5 \times 10^{-10}$ M) and maximal release at 16 ng ml⁻¹ ($\sim 6.7 \times 10^{-10}$ M) or higher. The FSH-releasing activity was inhibited by inhibin. Activin had no effect on the secretion of luteinizing hormone. The FSH-releasing

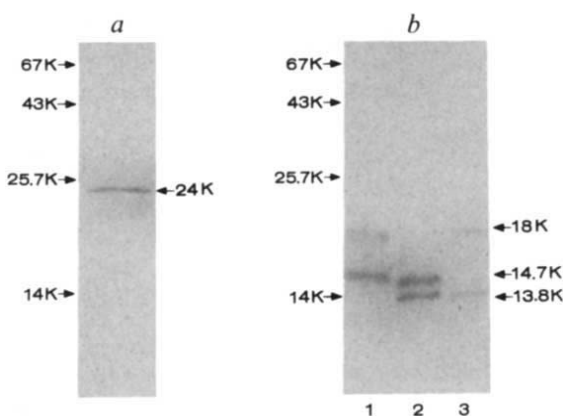


Fig. 2 SDS-polyacrylamide gel electrophoretic analysis of the purified activin under non-reducing (a) and reducing (b) conditions. Lanes 1 and 3 in b denote the purified inhibins A and B, respectively, being analysed simultaneously with activin (lane 2). The high- M_r band at 18K (18,000 M_r) is the common α -subunit of the inhibins and the low- M_r bands at 14.7K and 13.8K correspond to the β -subunits of inhibin A and inhibin B, respectively. The following M_r standards were used to calibrate the gel: bovine serum albumin (M_r 67K), ovalbumin (43K), α -chymotrypsinogen (25.7K) and lysozyme (14K).

Methods. The purified proteins were analysed in 1-mm-thick 15% polyacrylamide gels according to the method of Laemmli⁸. The proteins were revealed by a Coomassie blue staining reagent. For non-reducing conditions, 1 μ g of protein in 20 μ l of water was incubated with 20 μ l of sample buffer (0.125 M Tris-HCl, pH 6.8/20% (v/v) glycerol/4% SDS/0.04% bromophenol blue) for 1 h at 37 °C and then loaded onto the gel. Electrophoresis was at constant voltage (200 V) for 6 h at room temperature. For reducing conditions, 1.5 μ g of protein was incubated first with 20 μ l of 0.02 M dithiothreitol for 15 min at 37 °C, then 20 μ l of sample buffer was added and the incubation was continued for 1 h before the sample was applied to the gel. Electrophoresis was carried out as above except that 5 mM dithiothreitol was included in the electrophoretic buffer.

activity requires both subunits to be joined together; reduction of the disulphide bonds by dithiothreitol destroyed bioactivity (as in the case of inhibin¹).

Microsequencing of the intact activin on an Applied Biosystems model 470A protein sequencer revealed the first 32 N-terminal residues as shown in Fig. 3a. These sequence data are identical to the theoretical residues obtained if the two β -subunits of inhibins A and B were sequenced simultaneously (see Fig. 3b). To determine the structure of the two activin subunits, the molecule was compared with inhibins A and B by SDS-PAGE under reducing conditions. As shown in Fig. 2b, the migration of the two reduced subunits of activin coincides with that of the reduced β -subunits of inhibins A and B, respectively. Thus, based on the M_r s of the reduced subunits and their N-terminal sequences, we propose that activin, one of the FSH-releasing substances present in porcine follicular fluid, is a heterodimer comprised of the two β -subunits of inhibins A and B.

In our original report on the characterization of porcine follicular fluid inhibins², we had speculated, based on complementary DNA sequencing, that the β -subunits of inhibins A and B contain 116 and 115 amino acids, respectively. In view of the different sizes of the two β -subunits on SDS-PAGE analysis under reducing conditions (β_A slightly larger than the β_B), it is possible that the mature β_B undergoes proteolytic processing³ at the C-terminal Lys-Arg sequence of residues 101 and 102 to yield a β_B -subunit ~16 amino acids shorter than the β_A -chain. This would also be the case for the β_B -subunit of activin. We have noted previously² the striking homology between the β -subunits of the two forms of inhibins and TGF- β

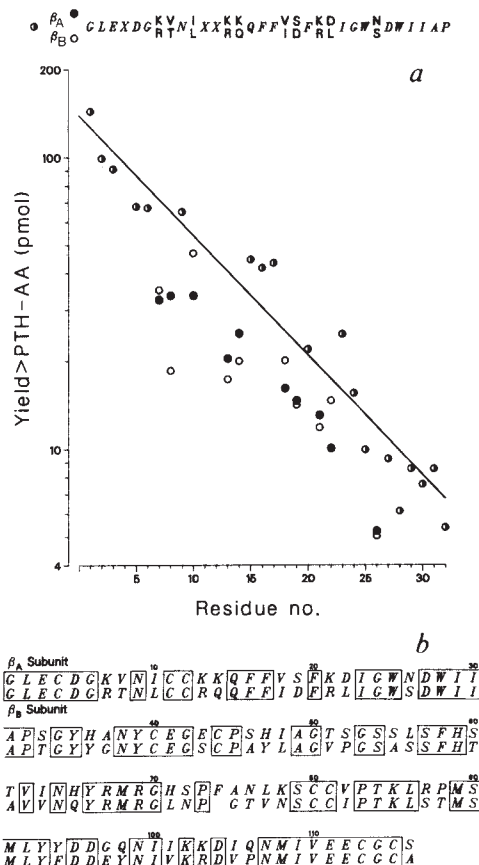


Fig. 3 a, N-terminal sequence analysis of activin. Residues determined that are identical to both subunits are denoted by ●. Unique residues present only in the β_A -subunit are designated by ● and those present only in the β_B -subunit by ○. An X denotes a residue (cysteine?) which is not identified. b, Primary structure of the β -subunits of inhibins A and B deduced from molecular cloning². The regions containing identical residues are boxed. A gap is introduced at position 74 of β_B to maximize homology alignment with β_A .

Methods. The protein (200 pmol) was sequenced using an Applied Biosystems 470A protein sequencer as described previously⁹ and the released phenylthiohydantoin-amino acids (PTH-AA) analysed by an online Applied Biosystems 120A PTH-amino-acid analyser. The initial yield was 36% and the average repetitive yield 91.1%.

and have recently shown that the homodimer TGF- β has intrinsic FSH-releasing activity⁴. To see FSH-releasing activity in a molecule comprised of the two β -subunits of inhibins A and B is thus in keeping with the TGF- β precedent. What is striking is that when each of the β -subunits of the inhibins is combined with an α -subunit, the resulting molecule inhibits the basal release of FSH, whereas if the two β -subunits are paired together, the resulting molecule acts as an FSH releaser. It is conceivable that a homodimer composed of either of the β -subunits of inhibin A or B could produce similar FSH-releasing activity. Such a precedent can be found in the case of platelet-derived growth factor (PDGF), a heterodimer of two closely related A and B subunits⁶, the same PDGF-like biological activity also being exhibited by a homodimer of either the A or B subunit⁷.

Our previous report² on the structure of inhibins A and B demonstrated that each subunit (α , β_A , β_B) is the product of a different messenger RNA rather than being processed from some unique, very large precursor, and we hypothesized the existence of multiple combinations of the inhibin subunits, including hybrids with subunits of the ubiquitous TGF- β . The characterization of activin as a heterodimer of the two β -subunits of

inhibins A and B reinforces that hypothesis. If such rearrangements of several gene products are the norm, rather than the exception, this process clearly considerably extends the diversity of the final products (and of their biological activity) derived from a limited number of genes. Multiple arrangements of protein subunits through disulphide bridges, leading to mature molecules with totally different biological activities (possibly of opposite biological activities, as here), would seem to extend to the gene products the possibility of reorganizations, as is now well recognized occurs in DNA and RNA.

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Inducible cellular transformation by a metallothionein-*ras* hybrid oncogene leads to natural killer cell susceptibility

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Natural killer (NK) cells are lymphoid effector cells possessing spontaneous cytolytic activity against a variety of tumour targets¹. The fact that NK cells pre-exist at high frequency and require no lengthy activation, and the observation that differentiated and metastatic tumour cells often have a decreased sensitivity to NK cytotoxicity have led to the hypothesis that these cells may be involved in the earliest stages of antitumour surveillance². Central to this model is the prediction that NK sensitivity must arise during cellular transformation. To test this prediction directly, we have constructed a vector containing the transforming gene from the EJ bladder carcinoma cell line under the transcriptional control of the mouse metallothionein-I promoter. When induced with heavy metal ions, mouse fibroblast lines containing this vector become dramatically sensitive to NK-mediated cytotoxicity concomitant with the expression of the cellular Harvey *ras* (c-Ha-*ras*) p21 protein and with cellular transformation.

To examine the NK sensitivity of cells during the transformation process, we have constructed vectors that permit controlled expression of c-Ha-*ras* (Fig. 1). For constitutive expression of *ras* in fibroblast cells, a 6.6-kilobase (kb) *Bam*HI fragment containing the transforming human c-Ha-*ras* gene³ (from the EJ bladder carcinoma cell line) under the control of its own promoter was introduced into the *Bam*HI site of pSV2neo (ref. 4) and called pREJ. For inducible *ras* expression, the mouse metallothionein-I promoter⁵ was ligated upstream from the coding sequence of the EJ *ras* gene. This zinc-inducible promoter has been used to express a number of heterologous genes both *in vitro*⁶ and *in vivo*⁷. The resultant chimaeric oncogene was introduced into pSV2neo and called pMTEJ.

Table 1 NK susceptibility using spleen cells as effectors

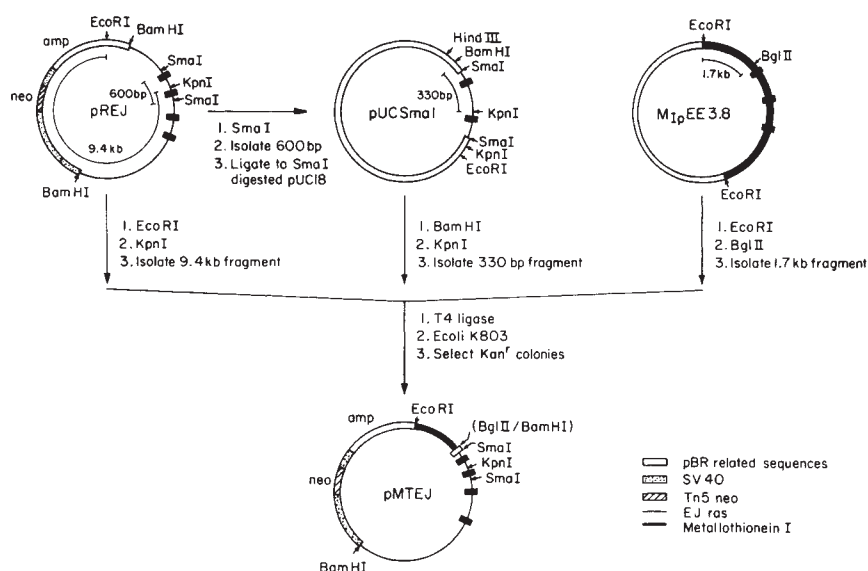
Target cells	Vector	Zn ²⁺ Induction	No. of expts	Mean cytotoxicity $\pm$ s.e.		P value
				% Lysis (200/1 E:T)	LU/10 ⁶	
10T1/2	—	—	4	10 $\pm$ 4	1.0 $\pm$ 0.3	NS
		+	3	17 $\pm$ 3	1.2 $\pm$ 0.3	
212	pMTEJ	—	15	18 $\pm$ 3	1.4 $\pm$ 0.4	<0.001
		+	17	57 $\pm$ 4	9.3 $\pm$ 1.2	
245	pREJ	—	4	46 $\pm$ 10	5.7 $\pm$ 1.7	NS
246	pREJ	—	3	33 $\pm$ 13	3.5 $\pm$ 1.5	
247	pREJ	—	5	38 $\pm$ 2	6.0 $\pm$ 1.2	NS
YAC1.2	—	+	1	40 $\pm$ 3	8	
		—	11	51 $\pm$ 4.5	19 $\pm$ 3.3	

Target cells were grown for 24-48 h in the presence or absence of 50 μ M zinc sulphate and collected by trypsinization. ⁵¹Cr-labelled cells were tested in a 6-h cytotoxicity assay against spleen cells pooled from 8-12-week-old CBA mice injected intraperitoneally on day -1 with 100 μ g of poly-I:C. Effector titration curves yielded maximum lysis values at a 200/1 effector:target ratio (E:T) as shown and lowest values at 1.5/1. One lytic unit (LU) was defined as the number of effector cells required to lyse 20% of the target cells, and the data are expressed as the mean $\pm$ standard error (s.e.) and LU per 10⁶ spleen cells in data pooled from 1-17 independent experiments. P values represent the probability (Student's *t*-test) that LU values for zinc-induced compared with uninduced cells are not different. NS, not significant.

The vectors were transferred into the murine fibroblast cell line C3H 10T1/2 (ref. 8) via protoplast fusion. Cells were selected for resistance to the antibiotic G418 (neo⁺) in the absence of zinc. All of the isolated clones were then tested for their responsiveness to zinc ions in the medium. Each line was plated in the presence or absence of 50 μ M zinc and their morphologies were compared. pSV2neo seemed to have no effect on colony morphology, leaving the cells flat and contact inhibited in the presence or absence of zinc. On the other hand, in 5 of the 10 neo⁺ pREJ colonies, the cells were rounded and more refractile, and within the colony the cells criss-crossed in a disorganized manner, with no evidence of contact inhibition. Again, zinc had no effect on the morphology of these lines. The vector pMTEJ gave rise to a broader spectrum of morphologies, including normal and fully transformed colonies and some intermediate morphologies not seen with pREJ. These exhibited contact inhibition but were more refractile and more densely crowded than the 10T1/2 parent cells or any pSV2neo lines. Of the 27 neo⁺ pMTEJ colonies obtained, 2 were fully transformed and 3 were of intermediate appearance. One of the 27 pMTEJ lines (212), which in the absence of zinc has an intermediate but nearly normal morphology, became highly refractile and formed an irregular criss-crossed pattern of fusiform cells 48-72 h after exposure to zinc (Fig. 2). In all cases, removal of zinc from the medium led to the return of the original morphology within 48 h, indicating that the continued expression of *ras* is required to maintain transformation.

Human *ras*-specific transcripts were detected in the RNA of all lines that exhibited a transformed morphology (data not shown), but inducible transcription was found only in the line showing zinc-induced morphological change. The 212 cell line expressed a low basal level of a 1.2-kb human *ras*-specific sequence not found in the 10T1/2 line (Fig. 3a). When induced with 50 μ M zinc sulphate, the expression increased six to eight-fold within 24 h to levels higher than those found in the pREJ line 245 or in the human bladder carcinoma line 5637. The slightly reduced size of this transcript compared with controls can be accounted for by the reduced size of the hybrid 5'UT relative to the predicted c-Ha-*ras* 5'UT⁹. RNA blots were washed and rehybridized with a probe specific for β_2 -microglobulin, confirming that equal amounts of RNA had been loaded in each lane. Consistent results were obtained when the levels of bio-synthetically labelled p21 proteins were examined by immunoprecipitation with monoclonal antibodies specific for c-Ha-*ras* p21¹⁰. The level of p21 increased dramatically in the

Fig. 1 Construction of Ha-ras expression vectors. The 6.6-kb *Bam*HI fragment from pEJ³ was subcloned into the *Bam*HI site of pSV2neo⁴ and the resultant plasmid was termed pREJ. From this a 602-base pair (bp) *Sma*I fragment containing all of the 5'UT as well as exons 1 and 2 was subcloned into the *Sma*I site of pUC 18¹⁷ such that the 5'UT was adjacent to the pUC 18 polylinker *Bam*HI site. A 322-bp *Bam*HI-*Kpn*I fragment from this plasmid was then ligated to a 9.6-kb *Eco*RI-*Kpn*I fragment from pREJ and a 1.7-kb *Eco*RI-*Bgl*II fragment from M1pEE3^{8,5}, containing the metallothionein promoter and messenger RNA cap site¹⁸ in the construction of pMTEJ. Vectors were transferred into the recipient fibroblasts via protoplast fusion. The cells were detached by trypsin treatment and fused to bacterial protoplasts in suspension as described elsewhere¹⁹ using a 40% polyethelene glycol 1000 (BDH)/10% dimethyl sulphoxide mixture. Cell lines resistant to 1 mg ml⁻¹ G418 were isolated and subcloned by limiting dilution.



inducibly transformed line 212 to levels considerably higher than those found in the constitutively transformed line 245 (Fig. 3b).

Anchorage-independent cellular transformation was assessed more quantitatively by examining the colony-forming ability of the cells in semi-solid medium. 10T1/2 cells had no detectable colony-forming ability when plated in medium containing 0.3% agar in the presence or absence of zinc. Constitutively transformed pREJ lines 245, 246 and 247 had agar plating efficiencies of 2–3% irrespective of zinc ions. The inducible line 212, however, formed colonies at 0.5% in the absence of zinc but increased to 10% in the presence of 50 μ M zinc sulphate. These data demonstrate that the cell line 212 is inducible for c-Ha-ras expression and, hence, for cellular transformation.

Cytotoxicity assays performed on these cell lines consistently demonstrated that an increased sensitivity to NK-mediated lysis correlated with expression of the transforming c-Ha-ras oncogene. The highly lytic NK clone LH49-IIIIF6 (ref. 11) lysed 20% of the uninduced 212 cells within 6 h at an effector to target ratio of 5:1, compared with >90% lysis after induction with zinc ions (Fig. 4). In addition, NK clones with intermediate or low lytic activities on the standard NK target (YAC 1.2 lymphoma) had the same rank order of activities on 212 induced with zinc.

Similar results were obtained using murine spleen cells as effectors. The parental line C3H 10T1/2 (H-2^k) was moderately resistant to NK lysis by spleen cells from H-2-identical CBA mice treated with the NK stimulating agent poly-I:C (Table 1). pREJ-transformed lines expressed a much higher sensitivity to lysis, corresponding approximately with levels of *ras* expression. Neither the transformed nor parental lines became more NK sensitive through growth in the presence of zinc. However, 212 cells grown in the presence of zinc became significantly more sensitive to NK cells than those grown in its absence, and the level of cytolysis approached that of the YAC tumour line. Equivalent results were obtained with spleen cells from mice not boosted with poly-I:C when assayed for 18 h.

The effector cells were further confirmed to be of NK origin by the observation that lytic activity against zinc-induced 212 was much reduced in spleen cells from NK-deficient beige mice¹². In addition, lytic activity could be completely abrogated by pretreatment of the spleen cells with complement plus anti-NK1.2 (ref. 13) or anti-asialo GM₁ (ref 14) antisera, indicating that the effector cells, like NK cells, bear these surface antigens (data not shown). In contrast, the T-helper-specific Lyt 1.1 antibody had no effect on this activity. The zinc-induced 212 cells were no more sensitive to lysis by 10T1/2-allo-specific or

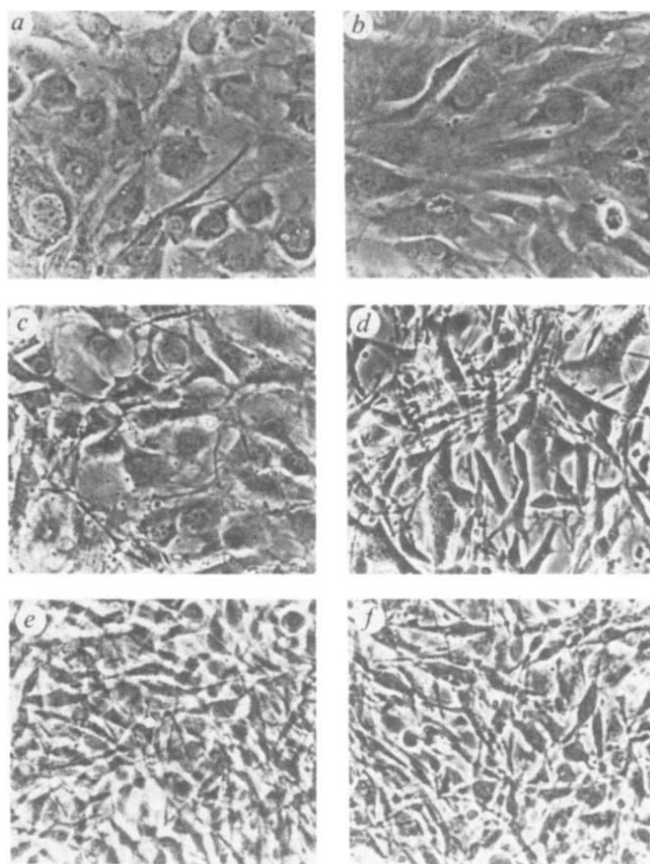


Fig. 2 Induction of morphological change with zinc. Cells were grown to confluence in medium containing 10% fetal calf serum with (*b, d, f*) or without (*a, c, e*) 50 μ M zinc sulphate, and photographed with phase contrast. Cell lines shown are 10T1/2 (*a, b*), the pMTEJ line 212 (*c, d*) and a pREJ line 245 (*e, f*). $\times 320$.

H-2^k-specific cytotoxic T lymphocytes than were uninduced 212. At a 6:1 effector/target ratio, the lysis of uninduced 212 cells by allospecific cytotoxic T cells was 62 ± 3% compared with 65 ± 5% for zinc-induced 212 cells. The lytic units per 10⁶ lymphocytes (as described in Table 1), calculated at 20% lysis, were 600 and 800, respectively. Similarly, anti-H-2^k-specific cytotoxic T cells from congenic resistant strains gave 49% lysis

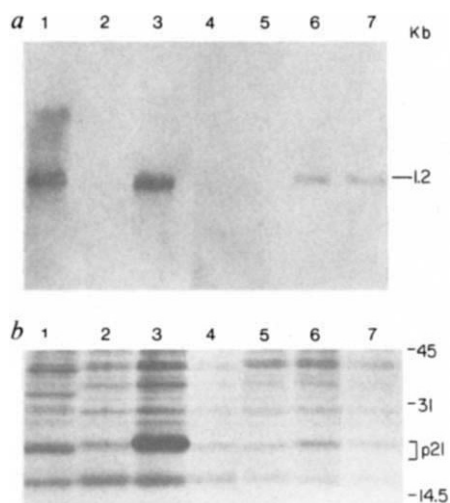


Fig. 3 Induction of *ras* expression in cell line 212. *a*, RNA blot analysis. Total cellular RNA was isolated² from cells cultured for 48 h in the presence (lanes 3, 5, 7) or absence (lanes 1, 2, 4, 6) of 50 μ M zinc sulphate in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum. RNA (20 μ g per lane) was electrophoresed and transferred to nitrocellulose as described previously²¹. Hybridization was performed as described elsewhere²¹ using as probe the 602-bp *Sma*I fragment from the plasmid pUC *Sma*I described in Fig. 1. The cell lines studied are as follows: 5637 (lane 1), 212 (lanes 2, 3), 10T1/2 (lanes 4, 5) and 245 (lanes 6, 7). *b*, p21 immunoprecipitation. Biosynthetic labelling was performed by incubating 50% confluent 60-mm cultures for 24 h in (DMEM) lacking methionine supplemented with 250 μ Ci ml⁻¹ ³⁵S-methionine (Amersham). Labelled cells were washed twice and lysed as described elsewhere¹⁰. Immunoprecipitation involved the addition of 5 μ l of ammonium sulphate concentrated YA6-172 rat monoclonal anti-p21 antibody¹⁰ to 300 μ l of lysate containing 10⁷ trichloroacetic acid-precipitable c.p.m. and incubation for 4 h at 4 °C. To this was added 25 μ l (as a 25% suspension) of protein A-Sepharose beads previously coated with rabbit IgG specific for rat IgG (Cappel) and incubated at 4 °C overnight. The beads were then washed four times in phosphate-buffered saline and four times in lysis buffer before electrophoresis on a 12.5% acrylamide slab gel²². The gel was soaked in Amplify (Amersham) before drying and exposure to XAR film (Kodak) at -70 °C. The loading order of this gel is identical to that for *a*. The relative molecular masses of the marker proteins are indicated on the right ($\times 10^{-3}$).

of 212 with no induction and 48% with zinc induction at 50:1 effector to target ratios, and the lytic units were the same at 16 lytic units per 10⁶ cells. Furthermore, trinitrophenyl (TNP)-haptenated, zinc-induced 212 cells did not lyse more readily with complement plus anti-TNP antibodies than did the unstimulated equivalents (data not shown), thereby demonstrating that zinc induction of 212 cells does not make them more sensitive to lysis in general.

These results demonstrate the potential for a single oncogene with a defined lesion to alter the NK sensitivity of the murine fibroblast cell line C3H 10T1/2. Previous studies with methylcholanthrene (MCA)-transformed BALB/c fibroblasts have demonstrated that the same effect can be acquired during transformation by chemical carcinogens¹⁵. The fact that MCA-transformed C3H 10T1/2 lines have been found to contain activated *ras* oncogenes¹⁶ suggests that MCA-induced NK sensitivity could result from mutation of the *ras* locus.

The ability to regulate transcription of the c-Ha-*ras* oncogene allows us to mimic the events occurring during cellular transformation. The low level of p21 expressed in uninduced 212 cells does not increase their NK sensitivity relative to the parental

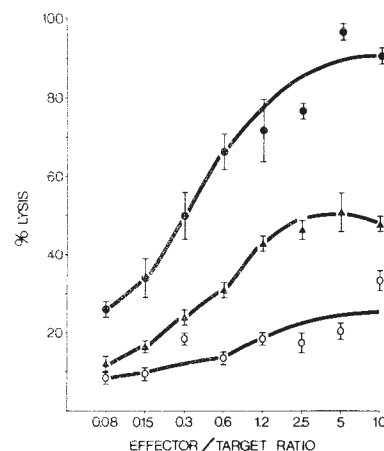


Fig. 4 Increased sensitivity to a cytolytic NK clone. The pMTEJ line 212 was grown for 24 h in the presence (●) or absence (○) of 50 μ M zinc added to the culture medium and collected by trypsinization. YAC 1.2 lymphoma cells (▲) or 212 cells were labelled for 2 h with ⁵¹Cr and tested in a 6-h cytotoxicity assay against a subclone (11IF6) of the C57Bl/6 NK clone (LH 49)¹¹ kindly provided by Dr Colin Brooks (Fred Hutchinson, Seattle). As reported for the original clone, the 11IF6 subclone was thyl⁺, aGM⁺, Lyt1.2⁺, Lyt2.2⁺, NK1.2⁺, Qa4⁺ as determined by immunofluorescence staining and flow cytometry. Values represent the mean % lysis $\pm$ standard error in triplicate wells. Spontaneous release was <10% of total label for the three targets. This experiment was repeated three times with similar results.

line 10T1/2 but, concomitant with zinc-induced transformation, the cells acquire an increased sensitivity to NK cytotoxicity which could make them targets for anti-tumour surveillance. This effect seems to be an early form of host defence, as other studies have found that tumour progression and metastasis are generally accompanied by the acquisition of NK resistance. Additional studies are under way to determine the molecular changes associated with *ras*-mediated NK sensitivity, and the generality of this phenomenon.

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Sequence organization and genomic complexity of primate $\theta 1$ globin gene, a novel α -globin-like gene

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The α -like and β -like globin genes have provided a paradigm for the study of molecular evolution and regulation of multigene families in eukaryotes. The human α -globin gene cluster, which is on chromosome 16 (ref. 1), consists of six genes arranged in the order²⁻⁵ 5'- ζ (embryonic)- $\psi\zeta$ - $\psi\alpha 2$ - $\psi\alpha 1$ - $\alpha 2$ (adult)- $\alpha 1$ (adult)-3'. DNA sequencing data have demonstrated that ζ (ref. 6) and $\alpha 2$ (or $\alpha 1$, refs 7-9) are the embryonic and adult genes, respectively, while $\psi\zeta$ (ref. 6), $\psi\alpha 2$ (ref. 5) $\psi\alpha 1$ (ref. 10) are all inactive pseudogenes. Restriction mapping analysis has shown that the structure of this locus in several anthropoid primates is nearly identical to that of the human^{11,12}. Recently, we have isolated the adult α -globin gene region from orang-utan, olive baboon and rhesus macaque by molecular cloning. We report here the complete nucleotide sequence of a gene located immediately downstream from the adult $\alpha 1$ -globin gene of the orang-utan, along with its flanking DNA. We designate this gene as $\theta 1$, and show that it contains the essential sequence elements required for an expressive gene. The putative polypeptide is 141 amino acids long, identical to that of the α - or ζ -globin, but its predicted amino-acid sequence is nearly as different from the orang-utan α -globin (55 differences) as the human ζ -globin is from the human α -globin (59 differences), suggesting an ancient history for the $\theta 1$ -globin gene. Results of blot hybridization experiments using the cloned orang-utan $\theta 1$ gene sequence as probe demonstrate a similar $\alpha 2$ - $\alpha 1$ - $\theta 1$ linkage map existing in the human genome. Furthermore, multiple copies of sequences homologous to the $\theta 1$ gene are detected in both human and orang-utan. These results cast a new light on the primate α -globin gene family, and have intriguing implications for the existence of previously unreported, functional globin-like gene(s) in the primate genomes.

The human α -globin cluster can be used to investigate the processes of gene duplication, insertion, and correction, in addition to the better-known molecular evolutionary processes¹³⁻¹⁶. To study these processes further, we have constructed genomic libraries of three non-human primates (orang-utan, *Pongo pygmaeus*; olive baboon, *Papio anubis*; rhesus macaque, *Macaca mulatta*), and screened with a 1.5-kilobase (kb) *Pst*I fragment containing the human $\alpha 1$ -globin gene. One of the positive recombinant phage clones, $\lambda\theta\alpha G1$, isolated from an orang-utan liver DNA library, contains duplicated functional adult α -globin genes, $\alpha 2$ and $\alpha 1$ ¹⁷ (see map in Fig. 1). Unexpectedly, blot hybridization and partial sequencing of the DNA region flanking the 3' side of the orang-utan $\alpha 1$ -globin gene revealed a previously unreported α -globin-like sequence¹⁷, which we now designate as $\theta 1$. The orang-utan $\theta 1$ gene contains all the sequence elements necessary for an expressible, protein-coding gene although its actual expression and function are unknown. However, due to its unique promoter organization and predicted amino-acid sequence, we designate it as $\theta 1$ following the suggestion of Dr George Stamatoyannopoulos.

Approximately 1.2 kb of DNA sequences containing and flanking the orang-utan $\theta 1$ gene have been analysed (Figs 1, 2). Like the adult $\alpha 1$ -globin gene, the $\theta 1$ gene contains three exons and two introns. The alignment of its sequence with the $\alpha 1$ -globin gene (Fig. 2) indicates that their exons are ~75% identical base-for-base without any deletion or insertion. The introns and most of the flanking sequences of the two genes, however, have diverged to the point of virtually random homology. Further-

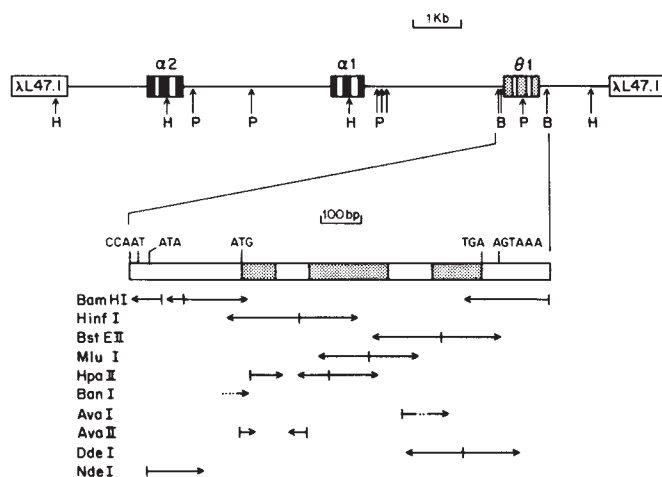


Fig. 1 Above, map of the adult α -globin gene region of the orang-utan. Restriction enzyme sites are as follows: P, *Pvu*II; H, *Hind*III; B, *Bam*HI. Below, sequencing strategy of $\theta 1$. Presumptive regulatory signals are given above the gene; presumptive coding sequences are stippled. The nucleotide sequences of the orang-utan $\theta 1$ gene and its flanking regions were determined by the method of Maxam and Gilbert²⁸. Restriction fragments sequenced were labelled with ³²P at their 3' ends by the Klenow enzyme. For the putative coding regions, both strands were sequenced.

more, despite the conserved lengths of the exons (exon 1, 95 base pairs (bp); exon 2, 205 bp; exon 3, 126 bp), the lengths of the introns and the untranslated regions of these two genes are significantly different. The sequence features that are required for the expression of protein-coding genes seem to be conserved within and flanking the $\theta 1$ gene. These include the upstream CCAAT and ATA boxes, the polyadenylation signal, the polyadenylation site, the splicing junctions, the initiation codons, the termination codon and an open reading frame without premature termination codons.

However, several features of these controlling elements differ between $\theta 1$ and the $\alpha 1$ -globin gene. First, whereas $\theta 1$ possesses upstream CCAAT and ATA boxes, the spacing between the two promoter elements is 29 bp, instead of 39 bp as in the $\alpha 1$ gene. Second, the ATA box of $\theta 1$ is located 157 bp further upstream from the ATG initiation codon. Third, the sequence between the two promoter boxes of $\theta 1$ is highly A+T-rich; in $\alpha 1$ the spacer sequence is G+C-rich. Interestingly the spacer region of each gene contains an inverted repeat which starts 7 bp downstream from CCAAT (CGCCCGG CCGGGCG in $\alpha 1$ and GTTTTACTAGAGACTAAAAAC in $\theta 1$). Fourth, the polyadenylation signal of $\theta 1$ is AGTAAA, rather than AATAAA. It is known that AGTAAA is functional in other gene systems¹⁸. Fifth, the termination codon in $\theta 1$ is TGA, instead of TAA as in $\alpha 1$.

If $\theta 1$ is indeed expressed, at a specific development stage in a specific tissue, it will code for a hitherto unknown α -like globin. The hypothetical polypeptide, whose primary sequence is given in Fig. 2, would be of the same length as α -globin, and bears no greater resemblance to the ' $\alpha 3$ globin' isolated from gorilla and chimpanzee¹⁹ than to the common $\alpha 1$ -globin of the orang-utan. The polypeptide would, despite the 55 amino-acid differences from orang-utan $\alpha 1$, nevertheless conserve: (1) all 5 amino acids (Thr 39, Phe 43, Leu 83, His 87, Tyr 140) invariant across all known haemoglobins and myoglobins; (2) all but one (Ser for Asn 97) of the 23 amino acids invariant across all known α -globins; and (3) 42 of the 50 amino acids invariant across all known mammalian α -globins²⁰. Of the eight sites not conserved when $\theta 1$ is compared with all other mammalian α -globins (Ala for Thr 38; His for Asp 85; Ser for Asn 97; Gln for Lys 99; Ser for Thr 134; Ala for Val 135; Ala for Thr 137; Gln for Lys 139),

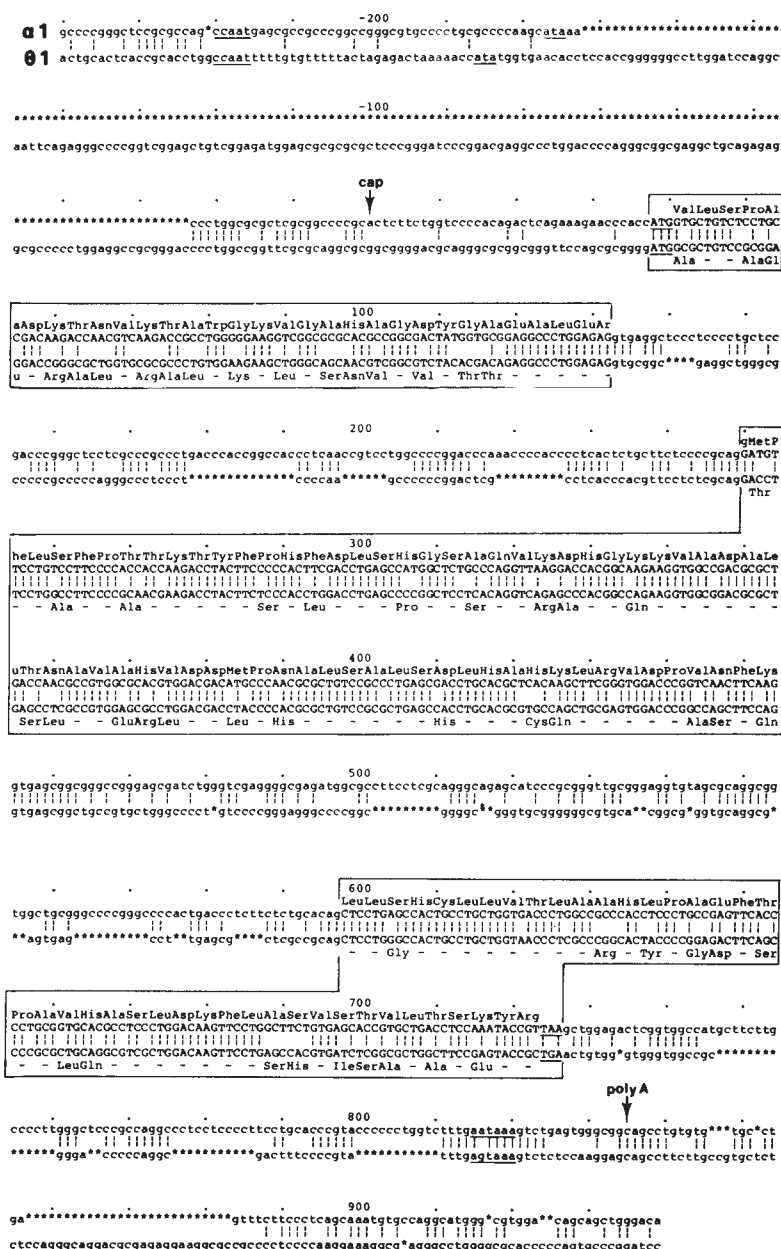


Fig. 2 Alignment of $\theta 1$ and $\alpha 1$ genes of the orang-utan. Sequence begins 20 bp upstream from the 'CCAAT' promoter and extends past the polyadenylation site of $\alpha 1$. Coding sequences are boxed and capitalized; noncoding sequences are given in small letters. The amino-acid translation is given above and below the coding sequence. That of $\alpha 1$ is given in full; where the amino acid in $\theta 1$ is identical, a dash is given below the nucleic acid sequence, otherwise the variant amino acid is given. Promoter, initiation codon, termination codon and polyadenylation signals are underlined. For the orang-utan $\alpha 1$ gene, cap and polyadenylation sites are indicated, based on the homologous sites in the human $\alpha 1$ globin gene. An asterisk indicates a deleted base in the corresponding position. Noncoding sequences are aligned to give maximum base-for-base homology, but these are probably overestimated and should be regarded with caution. Orang-utan $\alpha 1$ sequence is from ref. 17. The nucleotide at position 396 appeared as C when sequenced from the 5' direction, and as T when sequenced from the 3' direction. We give it as T for the sake of parsimony, as this is the same nucleotide as in $\alpha 1$; however, as a C the $\theta 1$ codon would change from CTG (Leu) to CCG (Pro).

four are in the carboxy-terminus. Two of these four sites, and eight other amino acids throughout the hypothetical $\theta 1$ protein, are different from those of the most common α -globin amino acid at the position, but identical to those of β -globin. These are Glu 5, Leu 13, Asn 20, Val 21, Ser 67, Leu 76, Gly 102, Gln 122, Ala 135 and Ala 137. A $\theta 1$ protein might thus have properties of both α - and β -globins.

We also note that another ATG codon lies 72 bp downstream from the ATA box of the $\theta 1$ gene. If we consider the possibility that this ATG triplet initiates the translation of $\theta 1$, three possibilities exist for a potential gene product. First, assuming no messenger RNA splicing, translation would be out of phase from α -globin and yield a protein of 126 amino acids until a termination codon is reached. Second, assuming splicing at identical points to the α -globins, out-of-phase translation would yield a protein of 208 amino acids. The last base of the termination codon in this case would be the first of the presumptive $\theta 1$ polyadenylation signal. Third, the mRNA might be spliced at different sites from α -globin, to yield a protein of unknown length. In connection with the latter two possibilities, we have not located an mRNA polyadenylation signal within 200 bp

downstream from the AGTAAA sequence (Fig. 2; J.M., results not shown).

Southern blot hybridization experiments have confirmed the genomic $\alpha 2$ - $\alpha 1$ - $\theta 1$ linkage in the orang-utan, and established a similar linkage in the human. The cloned orang-utan $\theta 1$ gene sequence hybridizes to a number of genomic restriction fragments of human, orang-utan, olive baboon and rhesus macaque (Fig. 3). Under the stringent conditions used for the blot hybridization (see Fig. 3 legend), none of the bands on the autoradiographs of human restriction digests corresponds to those fragments containing either the ζ -^{2,6} or the β -globin-like genes²¹. In the orang-utan digests (Fig. 3c), all of the restriction fragments containing the $\theta 1$ gene, as mapped in the phage clone $\lambda 0\alpha G1$ ¹⁷, were visible on the autoradiograph (the bands denoted by asterisks in Fig. 3c).

The restriction sites of *EcoRI*, *BglII*, *HindIII* and *HpaI* spanning the 3' region of the human α -globin gene cluster have been mapped previously^{2,22}. Using a cloned unique sequence located immediately downstream from the human $\alpha 1$ -globin gene as the hybridization probe, we have also established the existence of the 3-kb *PvuII* fragment located immediately down-

Fig. 3 Genomic blot hybridization of human, orang-utan, macaque and baboon DNAs with the orang-utan $\theta 1$ gene sequence. The lines and numbers on the left side of each panel indicate the lengths (kb) of the markers. Asterisks denote the bands corresponding to the restriction fragments containing the human or orang-utan $\theta 1$ gene located downstream from the $\alpha 1$ -globin gene. Their locations and sizes are shown in the maps in Fig. 4a and b. **a**, Southern blot of baboon, human, orang-utan or rhesus macaque DNA digested with *Bam*HI (B) or *Hind*III (H). **b**, Southern blot of human chromosomal DNA digested with pairs of the following restriction enzymes: *Bgl*II (Bg), *Hind*III (H), *Eco*RI (E), *Pvu*II (P) and *Sac*I (S). **c**, Southern blot of orang-utan chromosomal DNA digested with *Bam*HI (B), *Hind*III (H), *Pvu*II (P), *Bam*HI + *Hind*III (B + M), or *Bam*HI + *Pvu*II (B + P).

Methods. Chromosomal DNA samples were digested with different restriction enzymes, electrophoresed in a 1% agarose gel, blotted and hybridized²⁹ with the 0.9-kb *Bam*HI fragment containing the orang-utan $\theta 1$ gene (see map of Fig. 1). The probe was labelled with ³²P by nick translation³⁰. The blot was hybridized at 68 °C in a solution containing 0.75 M Na⁺ salt, and washed at 68 °C in 50 mM Tris-HCl (pH 7.5) and 1 mM EDTA.

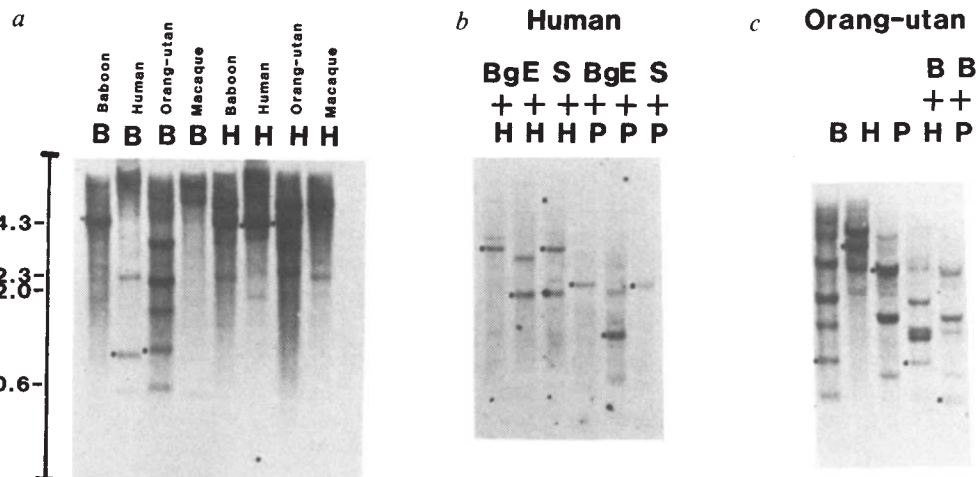
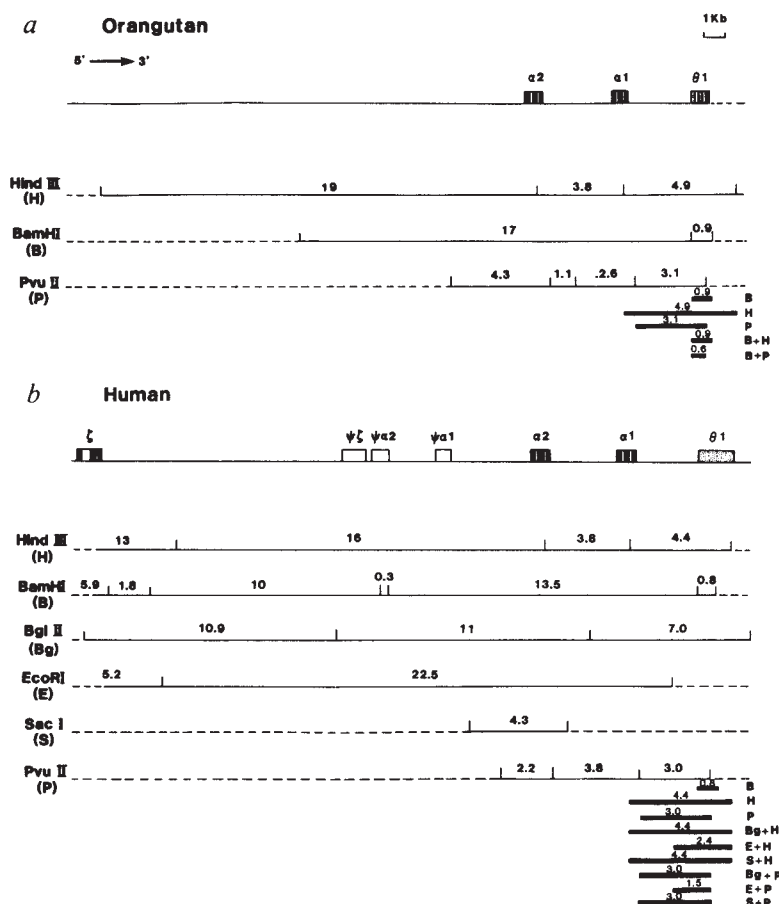


Fig. 4 **a**, $\alpha 2$ - $\alpha 1$ - $\theta 1$ linkage map in orang-utan. The arrangement of the $\alpha 2$, $\alpha 1$ and $\theta 1$ genes is derived from the study of Marks *et al.*¹⁷ and the blotting data in Fig. 3. The restriction maps of *Hind*III, *Bam*HI and *Pvu*II are shown below the gene linkage map. The genomic fragments that hybridize to the $\theta 1$ -specific probes, as denoted by an asterisk in Fig. 3, are represented by the black bars. The numbers above the individual fragments indicate their lengths in kb. **b**, Linkage map of the human α -globin-like gene cluster. The map is derived from refs 2, 5-10 and the present study (see text). The exons of the functional ζ , $\alpha 2$ and $\alpha 1$ are represented by black boxes while their introns are shown as blank boxes. The three pseudogenes $\psi\zeta$, $\psi\alpha 2$ and $\psi\alpha 1$ are represented by blank boxes and the $\theta 1$ gene by a stippled box. The restriction maps of the gene cluster are derived mainly from refs 2, 22 and the present study. The genomic fragments that hybridize to the orang-utan $\theta 1$ probe, as denoted by asterisks in Fig. 3, are represented by black bars below the restriction maps.



stream from the previously mapped 3.8-kb fragment (see Fig. 4; hybridization data not shown). The hybridization patterns of the human digests (Fig. 3a, b), combined with the restriction maps mentioned above, demonstrate that as in the orang-utan, a $\theta 1$ homologue also exists ~3 kb downstream from the $\alpha 1$ -globin gene. The $\alpha 2$ - $\alpha 1$ - $\theta 1$ linkage maps of orang-utan and human are shown in Fig. 4a and b, respectively.

In most digests shown in Fig. 3, there is more than one other band that cannot be explained by hybridization to the known genes of the α - or β -globin gene cluster. The presence of these bands (for example, the 2.7-kb band in the *Hind*III digests of

orang-utan DNA and the 1.0-kb *Pvu*II band in the *Eco*RI + *Pvu*II digest of human DNA) suggests that additional $\theta 1$ -homologous genes exist in the genomes of both species. However, due to lack of available restriction maps and probes, we do not know whether these are located further downstream from the $\theta 1$ gene. Further, the intensities of some of these additional bands, especially in the orang-utan, raise the interesting possibility that there are genomic regions where many $\theta 1$ -homologous sequences are tightly clustered together. These regions are being characterized by molecular cloning and sequence analysis.

Although we have not carried out detailed restriction mapping analysis for the macaque and baboon, the genomic hybridization pattern to the orang-utan $\theta 1$ probe (for example, see Fig. 3a) and partial sequencing analysis of a recombinant phage clone containing the baboon $\alpha 1$ gene and its 3'-flanking DNA indicate that a $\theta 1$ homologue also exists in these species on the 3' side of the $\alpha 1$ gene (J.P.S., J.M. and C.-K.J.S., unpublished results).

The $\alpha 2$ - $\alpha 1$ - $\theta 1$ linkage in human, orang-utan and the Old World monkeys suggests that the $\theta 1$ gene was generated by the tandem duplication of a primordial α -globin gene in a remote ancestor of these anthropoid primates. The duplication may even have antedated the emergence of the primates, since an α -globin-like pseudogene has been reported to exist downstream from the $\alpha 1$ gene of horse²³ and rabbit²⁴. In the former organism no sequence data are available²³; in the latter, no CCAAT and ATA boxes are found within 2 kb upstream from the gene ($\psi\alpha$; ref. 24), and several frameshift deletions in exon 2 create multiple premature termination codons. Nevertheless, there are several indications that the rabbit $\psi\alpha$ gene is indeed an orthologous counterpart of the orang-utan $\theta 1$ gene described here; for example, TGA instead of TAA as the termination codon, and AGTAAA instead of AATAAA as the polyadenylation signal. Further, exons of the rabbit $\psi\alpha$ and orang-utan $\theta 1$ resemble each other more than do orang-utan $\alpha 1$ and $\theta 1$ (Table 1). It thus seems that the $\theta 1$ gene has an ancient phylogenetic history in the mammalian genome.

If we postulate that $\theta 1$ is not, and has not in the recent phylogenetic past, been functional we may predict that the divergence between rabbit $\alpha 1$ and orang-utan $\alpha 1$, under the constraint of natural selection, will be considerably less than the divergence between rabbit $\psi\alpha$ and orang-utan $\theta 1$. While the noncoding segments are so different as to be unreliable in such a quantitative comparison, the percentages of amino-acid replacement mutations in the coding sequences may be compared, and this is done in Table 1. Insofar as the known func-

cation, Dr G. Stamatoyannopoulos for his suggestion of the $\theta 1$ designation, Candy Miller for typing the manuscript, Yerkes Regional Primate Research Center, Atlanta, for providing the orang-utan tissues, and the Primate Center of University of California, Davis, for the macaque and baboon tissues. C.-K.J.S. is supported by an NIH Research Career Development Award. *Note added in proof:* One of the human $\theta 1$ -globin-like genes has recently been cloned and mapped to chromosome 22 (J.P.S., T. Mohandas, and C.-K.J.S., manuscript in preparation).

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Table 1 Divergence for amino-acid replacement sites in $\alpha 1$ and $\theta 1$ homologues

Pairwise comparison	% Divergence replacement sites
Orang-utan $\alpha 1$ -rabbit $\alpha 1$	12
Orang-utan $\theta 1$ -rabbit $\psi\alpha$	13
Orang-utan $\alpha 1$ -orang-utan $\theta 1$	25
Rabbit $\alpha 1$ -rabbit $\psi\alpha$	27
Orang-utan $\alpha 1$ -rabbit $\psi\alpha$	29
Rabbit $\alpha 1$ -orang-utan $\theta 1$	29

Calculations were based on the method of Perler *et al.*²⁷ and data are from refs 17 and 24.

Assembly of microtubules at the tip of growing axons

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The growth of axons in the developing nervous system depends on the elongation of the microtubules that form their principal longitudinal structural element^{1,2}. It is not known whether individual microtubules in the axon elongate at their proximal ends, close to the cell body, and then move forward into the lengthening axon, or whether tubulin subunits are transported to the tip of the axon and assembled there onto the free ends of microtubules. The former possibility is supported by studies of slow axonal transport in mature nerves from which it has been deduced that microtubule assembly occurs principally at the neuronal cell body^{3,4}. By contrast, the polarity of microtubules in axons, which have their 'plus' or 'fast-growing' ends distal to the cell body⁵, suggests that assembly occurs at the growing tip, or growth cone, of the axon. We have addressed this question by topically applying Colcemid (*N*-desacetyl-*N*-methylcolchicine), and other drugs which alter microtubule stability, to different regions of isolated nerve cells growing in tissue culture. We find that the sensitivity to these drugs is greatest at the growth cone by at least two orders of magnitude, suggesting that this is a major site of microtubule assembly during axonal growth.

tional α -globin genes are only very slightly more similar between the two species (12% replacement site divergence) than are the new genes (13%), we interpret this as further indirect evidence for recent or current activity of this gene in at least one of the lineages. Partial DNA sequencing of a cloned baboon $\theta 1$ gene shows that its putative promoter sequences and coding regions are highly homologous to the orang-utan $\theta 1$ reported here (J.M., J.-P.S. and C.-K. J. S., unpublished results).

To ascertain that the $\theta 1$ globin gene is functional requires extensive gene transfer experiments, and a search for its corresponding mRNA and protein *in vivo*. Our preliminary studies have indicated that, like the human embryonic ζ -globin gene²⁵, the promoter of the orang-utan $\theta 1$ gene functions poorly in HeLa cell extract or in transfected Cos cells. Finally, it may be of interest that as yet uncharacterized globin polypeptide(s) exist during the early stages of human embryo development²⁶.

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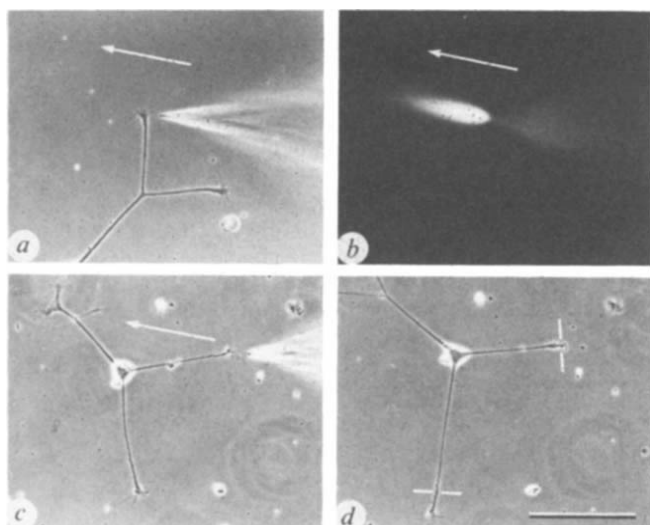


Fig. 1 Response of a growth cone to topically applied Colcemid. Dorsal root ganglia from 8–10-day-old chick embryos were trypsin-dissociated and cultured as described previously¹⁷. Cultures were mounted on the stage of an inverted Zeiss IM35 fluorescence microscope and kept at 37–38 °C by an air-stream incubator. A peristaltic perfusion pump circulated culture medium (total volume 25.6 ml) at a rate of 0.4–1 ml min⁻¹. Dow-Corning silicone fluid (1 ml) was applied to the culture to minimize evaporation. The outflow port of the perfusion system was adjusted so that the direction of flow of the medium (arrow) carried the drug across the cell of interest and removed it from the bulk culture medium. Glass capillary micropipettes (pulled to give tips 1–3 µm in diameter) were back-filled with the desired drug solution, containing 25–50 µg ml⁻¹ fluorescein. The micropipette was then placed at the desired point (a) and the flow of the drug solution, monitored by the fluorescence of the internal fluorescein marker, applied to the cell (b). (For clarity, the micropipette in this panel was raised slightly from the culture substratum and contained a higher concentration of fluorescein.) A typical response of a growth cone to the application of 0.1 µg ml⁻¹ Colcemid from a micropipette is shown at zero time (c), and after 30 min of application (d). Only that growth cone to which the drug was applied ceased its advance over the culture substratum. Scale bar, 100 µm.

Application of drug-containing solutions to localized regions of growing nerves was achieved by means of a pressure-driven micropipette and a background flow of medium to restrict diffusive spread. The localized region of application covered an area ~30 µm in diameter (Fig. 1a, b). Careful placement of the pipette and adjustment of the background flow allowed us to centre the region of drug application to the growth cone, neurite fibre or cell body.

A typical response of a growth cone exposed to 0.1 µg ml⁻¹ Colcemid applied through a micropipette is illustrated in Fig. 1c, d. The treated growth cone stops its advance within a few minutes. At the same time, a sister neurite from the same cell, but not exposed to the flow of drug-containing solution, continues unabated growth. We observed similar results for sister growth cones from a branched neurite.

A dose-response curve for the application of Colcemid to the growth cone and to the cell body is shown in Fig. 2. A concentration of Colcemid of 0.5 µg ml⁻¹ (1.35×10^{-6} M) caused significant inhibition of neurite growth when applied to the growth cone whereas 100 times this concentration had little effect on neurite growth when applied to the cell body.

We tested two other microtubule-active drugs, nocodazole⁶ and taxol⁷, for their effects on axonal growth in our system (Table 1). Although complete dose-response curves were not obtained, these agents were similar to Colcemid in that they were much more effective when applied to the growth cone than to the cell body. Application of either nocodazole or taxol to

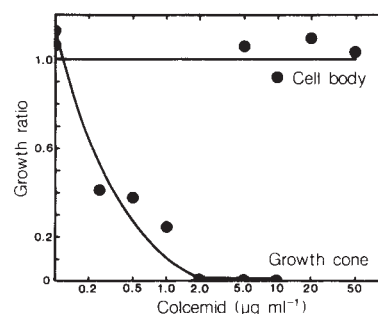


Fig. 2 Dose-response curve for the application of Colcemid to the growth cone and to the cell body. The drug was dissolved in culture medium without methylcellulose and containing 25 µg ml⁻¹ fluorescein and 10% Ficoll. These solutions were sonicated and filtered through 0.22-µm filters. Neurite outgrowth in control cultures was unaffected by addition of 50 µg ml⁻¹ fluorescein or 10% Ficoll. The response shown here is the ratio of the increase in length over a 20-min period following the application of the drug to the increase in length during 20 min before drug application. Drug was applied for 20 min. An average of 7 cells were observed per point with a minimum of $n = 4$ except for the growth cone application of 2 µg ml⁻¹ ($n = 3$) and 10 µg ml⁻¹ ($n = 1$). Neurites which retracted were scored as zero.

the growth cone inhibited axonal growth at concentrations at least 150 times lower than those needed to arrest axonal growth when applied to the cell body or neurite fibre. Lumicolcemid, a derivative of Colcemid which no longer binds to tubulin, had no effect on axonal growth when applied to the growth cone (Table 1).

In another series of experiments the time-course of axonal growth was followed for 30 min during drug applications after measurement of growth over a 30-min control period. Continual readjustment of the position of the micropipette was often required in these experiments when growth-cone application was used, as the neurites often retracted or grew beyond the point of application.

Typical time-courses showing the response of growth cones to Colcemid are shown in Fig. 3. A concentration of 2.7×10^{-7} M (0.1 µg ml⁻¹) applied to the growth cone arrests its advance within minutes, whereas a 10-fold higher concentration of this drug (2.7×10^{-6} M, 1 µg ml⁻¹) causes rapid retraction. Again, application of Colcemid at high concentration (4.0×10^{-5} M, or 15 µg ml⁻¹) to the neurite fibre or cell body had no detectable effect on the progress of the elongating axonal tip (Table 1).

Table 1 Concentrations of drugs required to inhibit axonal growth

Drug	Site of application		
	Growth cones (µg ml ⁻¹)	Neurites (µg ml ⁻¹)	Cell body (µg ml ⁻¹)
Colcemid	<0.1	>15	>15
Taxol	<0.1	>15	>15
Nocodazole	<0.1	>5	>15
Lumicolcemid	>5	ND	ND

The concentrations of Colcemid, taxol and nocodazole listed were enough to halt growth cone advance within 10 min of application to the growth cone but had no inhibitory effect on growth when applied to neurites or the cell body. ND, not done. Lumicolcemid was prepared from Colcemid by irradiation of an ethanol solution at 350 nm (ref. 16). Lumicolcemid, nocodazole and taxol were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1–5 mg ml⁻¹ and dilutions prepared as described in Fig. 2 legend. Neurite outgrowth in control cultures was unaffected by 1.5% DMSO (the maximum concentration in any application solution).

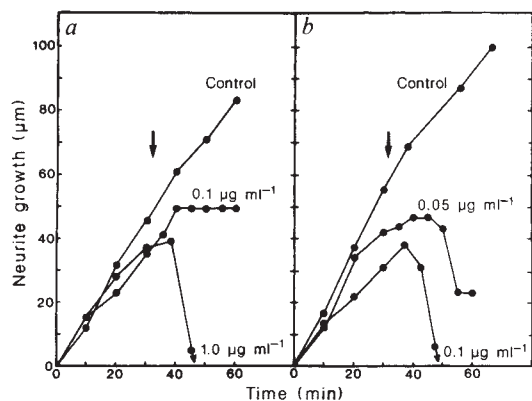


Fig. 3 Time-course for the response of growth cones to Colcemid when applied locally (a) or to the whole cell (b). The drug was applied at the time indicated by an arrow. Application of the drug to the whole cell was achieved by perfusion of the entire culture.

It was of interest to compare the effect of topically applied drugs, restricted to a small region of the nerve cell, with that of the same drug applied in the bathing medium to the entire surface of the growing nerve cell. Earlier studies of the inhibition of axonal growth by drugs added to the culture medium have shown effects within 6–24 h with as little as 2.5×10^{-8} M ($0.01 \mu\text{g ml}^{-1}$) colchicine⁸ or 7×10^{-8} M ($0.06 \mu\text{g ml}^{-1}$) taxol⁹. On a shorter timescale (Fig. 3), a significant inhibition of neurite growth was observed at Colcemid concentrations somewhat lower than those which inhibited growth when applied to the growth cone. But, taking into account the dilution of the drug which must occur when it is delivered by micropipette, the inhibitory effects of antimetabolic agents on neurite outgrowth probably arise from their effect on the growth cone, the most sensitive site.

The primary target of colchicine and related drugs is the assembly of microtubules^{10,11}. Therefore, our finding that substoichiometric concentrations of Colcemid and nocodazole inhibit nerve growth only when they are applied to the growth cone, and not when applied to the cell body or along the length of the axon, suggests that the growing tip of the axon is a major site of microtubule assembly in growing nerve axons. Taxol, on the other hand, promotes microtubule assembly by stabilizing microtubules⁷ and cause growth inhibition through an uncontrolled assembly of microtubules at the growth cone⁹. Thus, the controlled assembly of microtubules appears to be tightly coupled to nerve growth, a finding consistent with a recent report suggesting that microtubule assembly proteins are vital regulatory factors in neurite outgrowth from PC12 cells¹². Although it could be argued that the difference in sensitivity of growth cone and cell body arises because one is much larger than the other, or more active in the uptake of externally applied agents, we are reassured by the more than 100-fold difference in drug sensitivity of these two regions. Moreover, the rapid effects of Colcemid and nocodazole on mitosis show that they readily gain access to microtubules in the cytoplasm of even very large cells^{13,14}. Another possible objection is that even though an antimetabolic drug is applied to the growth cone, it might act at another site in the cell, being carried there by axonal transport. Against this objection we have clear evidence of restricted action of a topically applied drug (Fig. 1c, d).

If we are correct in thinking that microtubules assemble at the growing tip of an axon, then tubulin molecules must be available there in an unpolymerized form. Conceivably, tubulin could be polymerized by a Colcemid-resistant mechanism in the cell body, transported through the axon as microtubules and then selectively disassembled near the growth cone to provide

subunits for new assembly. But, it seems simpler to propose that tubulin is transported in an unassembled form and that most microtubules in the axon are as stationary as neurofilaments appear to be¹⁵. The nature of this tubulin-containing precursor, the mechanism by which it is carried in the axon and the regulation of its addition to the ends of microtubules are interesting and intriguing questions.

The idea for these experiments arose in a conversation with Dr Tim Mitchison, whom we thank. J.R.B. is on sabbatical leave from the Department of Biochemistry, Colorado State University, Fort Collins, CO 80523, USA and supported in part by a Senior International Fellowship, Fogarty Centre and NIH grant GM35126.

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Template-directed oligonucleotide ligation on hydroxylapatite

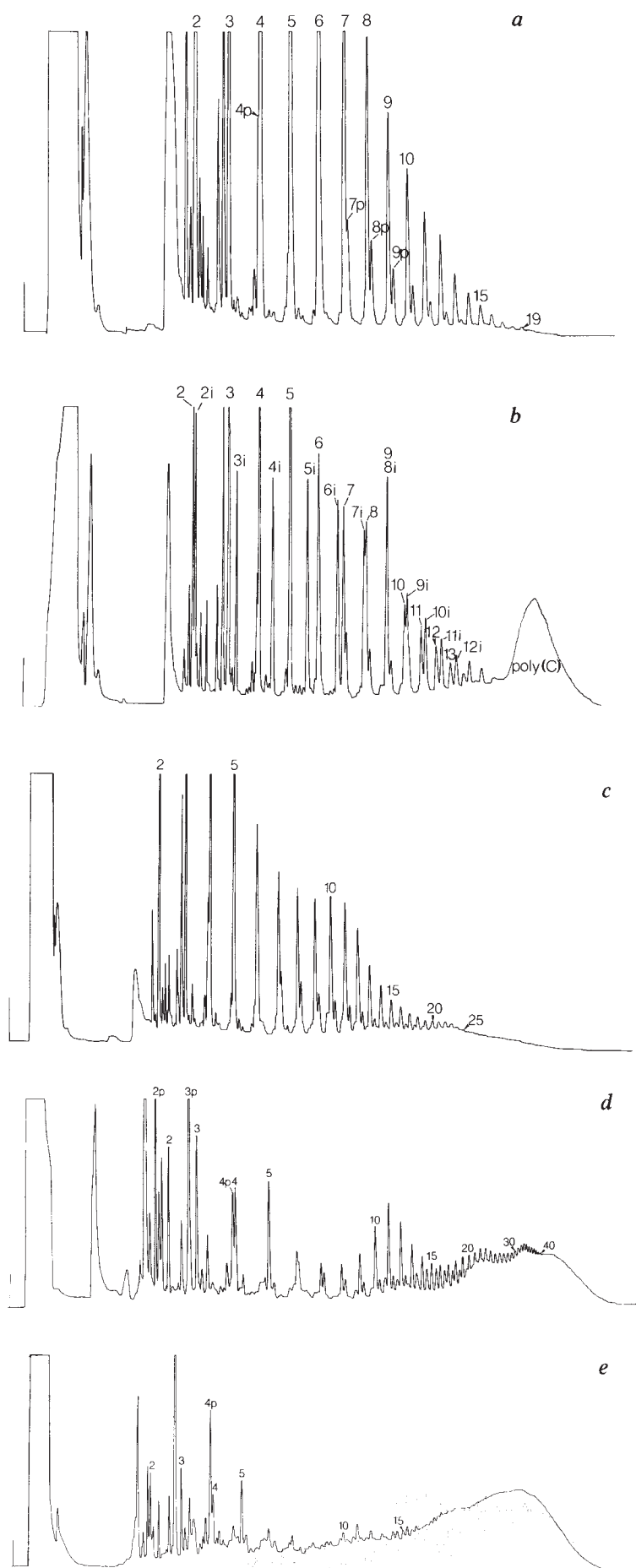
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Bernal¹, and subsequently other authors², have suggested that the prebiotic synthesis of the precursors of biopolymers could have occurred on a solid surface such as that provided by clay or some other mineral. The separation of products from the other components of the reaction mixture in such a system is reminiscent of modern solid-phase synthesis of polypeptides and polynucleotides. One such scheme envisages that growing polymers were localized by adsorption to a mineral surface where an activating agent or activated monomers were supplied continuously or cyclically^{3,4}. We have been trying to test this scheme using reactions which we believe may be related to those that occurred during prebiotic evolution. We have already reported that oligonucleotides adsorbed onto hydroxylapatite provide suitable templates for the oligomerization of (guanosine 5'-phosphor)-2-methylimidazole (2-MeImpG)^{5–7}. However, this is not a suitable test reaction, as 2-MeImpG oligomerization proceeds almost to completion in a single step. Here we report that a sequence of reactions in which initially formed oligo(G)s are reactivated by conversion to phosphorimidazolides in the presence of poly(C) and then allowed to ligate is ideal, in that repeated cycles can be carried out on the surface of hydroxylapatite, whereas in the liquid phase the cycle could be achieved only with considerable difficulty.

To obtain suitable substrates for the reaction, we adsorbed poly(C) (2.5 μmol) on hydroxylapatite (60 mg) and then added 2-MeImpG (7.5 μmol). The pattern of products obtained⁷ after incubation for 1 week at 0 °C is shown in Fig. 1a. Each reaction

Fig. 1 HPLC elution profiles of oligoguanylates on hydroxylapatite. *a*, Products of standard oligomerization of 2-MeImpG on poly(C): poly(C) (2.5 μ mol) adsorbed on 60 mg hydroxylapatite (BIO-GEL HTP; Bio-Rad Laboratories) and treated with 0.15 M 2-MeImpG (7.5 μ mol, 0.5 μ Ci), 0.2 M $MgCl_2$, 0.2 M NaCl, 0.1 M HEPES, sodium salt buffer, pH 8.0, in a 50 μ l volume (incubation for 7 days at 0 °C). *b*, Products of reactivation of oligoguanylates. Reaction mixture contained 1.0 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl and 1.0 M 2-methylimidazole, pH 6.0 (25 °C, 4 h, 200 μ l volume over hydroxylapatite). The ratio of the heights of N_i to $N_i + N$ gives the percentage reactivation. *c*, Products of one cycle of reactivation-ligation. Reaction mixture contained 0.2 M $MgCl_2$, 0.2 M NaCl, 0.5 M HEPES buffer (20 h, 37 °C, 200 μ l volume over hydroxylapatite). *d*, Products of four cycles of reactivation-ligation. *e*, Products after 16 cycles. The dotted curve is the elution profile of a 'high- M_r ' sample of poly(G) (Sigma). The numbers above selected peaks are the lengths of the corresponding oligomers; phosphorimidazolidines are indicated by the letter i; pyrophosphate-terminated oligomers are indicated by the letter p.



cycle is begun by centrifugation, decantation of the supernatant and addition of 200 μ l of 1 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in 1 M 2-methylimidazole buffer at pH 6 and 25 °C. After 4 h, the suspension is chilled (0 °C) and centrifuged, and the hydroxylapatite then separated from the supernatant. The ligation phase is initiated by adding 200 μ l of a solution containing 0.2 M NaCl, 0.2 M MgCl₂ and 0.5 M HEPES buffer at pH 8. Ligation is allowed to proceed at 37 °C for 20 h, after which the supernatant is removed and the next cycle of reaction initiated as above.

The oligonucleotides, together with the poly(C) template, are eluted from the hydroxylapatite using a solution of 0.5 M EDTA, 0.5 M K₄P₂O₇. When necessary, an aliquot of this wash is incubated with pancreatic ribonuclease at pH 7.6 to hydrolyse the poly(C) template. HPLC analysis on an RPC-5 column using a gradient of sodium perchlorate at pH 12 is carried out as reported previously⁷ to provide a profile of the products eluted from the hydroxylapatite.

Figure 1b shows the products obtained immediately after the first reactivation step; ~45% of the oligo(G)s had been converted to phosphorimidazolidines. Subsequent analyses at later stages of the experiment showed that a similar degree of reactivation was achieved in each cycle.

After one cycle of reactivation and ligation (Fig. 1c) short oligomers were depleted and longer ones enriched—oligomers in the size range 20–25 units were present only after ligation. Each successive cycle resulted in a further emphasis of the peaks corresponding to long oligomers at the expense of those corresponding to shorter ones. After four cycles of reactivation and ligation (Fig. 1d), peaks corresponding to oligomers up to 30–40 units long were resolved, together with an unresolved tail, found reproducibly, corresponding to oligomers at least 60 residues long. After 16 cycles almost all of the input oligo(G)s had been converted to material of high relative molecular mass (M_r) (Fig. 1e). The elution profile of a commercial sample of 'high- M_r ' oligo(G)s is included in Fig. 1e for comparison. Oligo(G)s adsorbed on hydroxylapatite in the absence of poly(C) were not detectably ligated after four cycles.

The yield of oligomers obtained after several ligation cycles does not decline steadily with increasing oligomer length, but passes through maxima at lengths of 11, 22 and 33 residues. Rhodes and Klug showed that adsorption on calcium phosphate leads to the cleavage of DNA by DNase I, yielding oligomers corresponding to integral numbers of turns of the DNA double-helix⁸. Our observations may have a related explanation, but the details are unclear.

To confirm that the broad absorption peak in the elution profile is due to oligonucleotides, we repeated the experiments described above using [8-¹⁴C]-MeImpG (specific activity 0.06 μ Ci/ μ mol⁻¹). After four cycles of reactivation and ligation, the products were applied to an RPC-5 column and fractions of the eluate corresponding to different ranges of oligomer length were collected. The activity of each fraction was determined in

Table 1 Distribution of radioactivity by oligoguanylate length

Range of oligomer length (no. of residues):	2–6	7–12	13–19	>19
Before ligation*	73.5†	20.9	4.4	1.2
After four ligation cycles*‡	32.7	23.1	16.9	27.3

* See legend to Fig. 1a for details.

† Values represent the c.p.m. in each fraction as a percentage of the total c.p.m. in the sample.

‡ See legend to Fig. 1b for details.

a liquid scintillation counter and the percentage of G residues present in each fraction calculated (Table 1). The radioactivity in each sample of the eluate correlated well with the ultraviolet intensity in the appropriate region of the elution profile; this shows that almost all the observed ultraviolet absorption of the HPLC eluate is due to G residues.

We next compared the radioactivity in each region measured for the multiply reactivated sample with that in the same region for a control sample. In the reactivated sample, we found that ~25% of the G residues were present in oligomers more than 20 residues long, while only 1% was present in oligomers of this size range for the control sample. The total recovery of radioactivity after four cycles was ~75% of the original input.

To establish that the products of ligation are 3'-5'-linked oligo(G)s, we used ribonuclease T₁, an enzyme that degrades 3'-5'-phosphodiester bonds only on the 3' side of G residues. The radioactively labelled product mixture was subjected to paper chromatography in *n*-propanol/ammonia/water (55:10:35 v:v); the material at the origin of the chromatogram included all oligo(G)s $\geq$ 4 units long. The region at the origin was cut out and degraded with RNase T₁; as anticipated, the only products were Gp, G, pGp, and a small amount of GppGp from pyrophosphate-'capped' chains. This result shows that the condensation is regiospecific and that no C residues were present in our products.

We do not mean to suggest that 2-MeImpG or water-soluble carbodiimides played any part in the origins of life on Earth. Our experiments show only that minerals lining a pond or tide pool could have provided an ideal site for the template-directed elongation and ligation of oligonucleotides in cyclic processes analogous to solid-phase synthesis. It would be premature to try to identify the activating agents or activated intermediates that were important on the primitive Earth; obvious suggestions include cyanamide and related compounds or inorganic trimetaphosphate as activating agents and nucleoside polyphosphates as activated intermediates.

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Focusing on hormone release

from L. Stephen Frawley, F.R. Boockfor and James P. Hoeffler

Reverse haemolytic plaque assays make possible the microscopic visualization of hormone release at the single-cell level. The method can quantify functional differences among hormone secretors of a given type.

PITUITARY cells that secrete the same hormone do not comprise a homogeneous population but differ from one another according to several morphological and functional criteria. Because of this high degree of heterogeneity, analysis of hormone release at the single-cell level may provide information more meaningful than that derived by evaluating the average response of an entire population of cells.

Such analysis was recently made possible by an adaptation of standard immunological methodologies^{1,2}. The technique for analysing single-cell secretions is known as the reverse haemolytic plaque assay and is based on antibody-directed, complement-mediated lysis of indicator erythrocytes in proximity to the hormone secretors (Fig. 1). The assay involves incubating secretor cells with indicator cells in a monolayer. One type of hormone secretor can be distinguished from another because, in the presence of hormone-specific antiserum and complement, it will induce the formation of microscopically identifiable plaques (zones of haemolysis).

In this review, we will summarize our application of plaque assays to endocrine problems and provide examples of experimental findings that demonstrate the power of the technique.

Heterogeneity

The standard version of the plaque assay (Fig. 1), though conceptually simple, is extremely versatile in terms of the information it provides about hormone secretion. Quantification of hormone release is generally achieved by one of two methods. First, the rate at which plaques form can be used as an index of the rate of hormone release because a threshold amount of hormone is required to induce plaque formation; cells that release more hormone molecules per unit time form plaques faster than those which release fewer molecules³. Alternatively, the sizes of plaques that develop around individual secretors provide a more cumulative index of the relative amount of hormone released^{2,4,5}.

Both of these simple methods of quantification have yielded valuable information concerning functional differences among hormone-secreting cells of a given type. For example, discontinuities in the rate of plaque formation among mammatropes

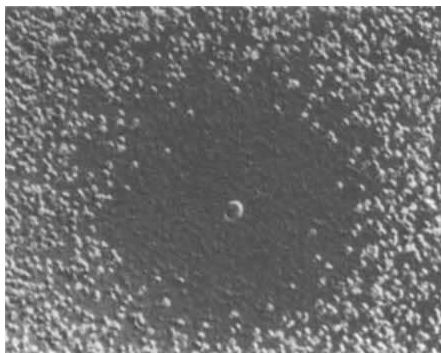


Fig. 1. In this standard plaque assay, mono-dispersed pituitary cells and protein-A coated ovine erythrocytes are infused into an assay chamber (constructed from a glass coverslip attached to a microscope slide with two pieces of double-sided sticky tape). The monolayer that forms is then incubated with hormone-specific antibody and complement. Hormone released from a pituitary cell binds to antibody which in turn attaches to protein-A on the erythrocyte surface. This facilitates complement fixation, lysis of the indicator cell, and the formation of haemolysis zones or plaques. Shown in this high magnification photomicrograph is a single PRL-releasing cell surrounded by a plaque in which ghosts of the lysed erythrocytes are apparent.

revealed that only a small fraction of these cells are maximally responsive to the inhibitory influences of hypothalamic dopamine or to the autosuppressive effects of prolactin (PRL) itself⁶. Likewise, stimulation of pituitary cells with growth hormone (GH) releasing factor shifted the frequency distribution of plaque sizes from unimodal to bimodal, suggesting that not all somatotropes are responsive to this secretagogue⁴.

The plaque assay also yields useful information when combined with immunocytochemistry. Studies of this type have shown that subpopulations of hormone secretors exist which contain hormone but do not release it⁷, or conversely, release hormone without storing appreciable quantities⁷. Taken together, these examples illustrate the utility of the standard plaque assay for characterizing functional differences among pituitary cells that release the same hormone.

Dual hormone release

By sequentially performing plaque assays for different hormones, it is possible to identify cells that release more than one hormone. The essence of the sequential plaque assay is that only the pituitary cells are attached to the chamber floor; fresh assay components (including indicator

cells) are added at the beginning of each sequence. Reidentification of plaque formers is facilitated by using a photoengraved coverslip as the roof of the incubation chamber. This assay has been employed in our laboratory to challenge the prevailing view that GH and PRL are invariably secreted by separate and distinct cell types in rats. We found that in male rats approximately one-third of all GH or PRL secretors actually release both hormones concurrently⁸. Moreover, *in vitro* treatment of these cells with oestrogen causes striking shifts in the ratios of single and dual hormone secretors, indicating these cell types may be functionally interconvertible⁹.

We have also employed this assay to monitor the ontogeny of GH and PRL secretors in rats and have observed that the vast majority (98 per cent) of initial PRL cells also release GH¹⁰. Inasmuch as GH cells precede PRL secretors during development, these results suggest that the former cell type may give rise to the latter. Thus, the sequential plaque assay provides a powerful tool for identifying and quantifying changes in the proportions of cells that release more than one hormone.

Plaque bioassay

While there is compelling evidence that PRL cells differ greatly in the amount of immunoreactive hormone released, the question of whether they are also heterogeneous with respect to the biopotency of secreted hormone has not been resolved. To address this issue, we developed an assay based on the ability of PRL to induce a dose-related release of casein from cultured mammary cells¹¹. We call this system the casein plaque bioassay because casein release is quantified by a standard plaque assay for the milk protein.

This system is sensitive enough to assess the bioactivity of PRL released by individual pituitary cells in microwell culture (Fig. 2). In addition, by removing the medium bathing an individual pituitary cell for bioassay and then subjecting the same cell to a standard plaque assay for PRL, it is possible to evaluate both the bio- and immuno-potency of hormone released from one cell.

Using this approach, we were surprised to find that many cells which formed large PRL plaques exhibited low biopotency, and vice versa¹¹. These findings show that

Endocrinology outlook

This week's review is aimed at the endocrinologist's needs. Selections include microplate manipulators, probes for chemical labelling and microtomes for sample slicing.

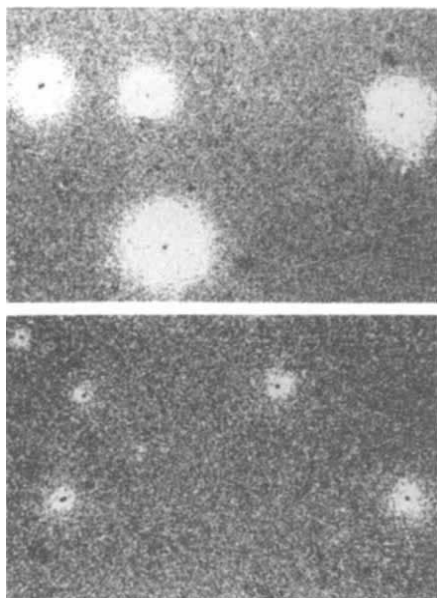


Fig. 2 The casein plaque bioassay is performed by pre-incubating monolayers of collagenase-dispersed mammary cells and indicator erythrocytes in either control medium or medium that previously bathed an individual PRL secretor. The monolayers were then subjected to a standard plaque assay for casein. This low magnification micrograph illustrates the sensitivity of this bioassay; mammary cells exposed to PRL released from a single pituitary cell (top panel) formed larger plaques than controls (bottom panel).

individual cells release forms of PRL which differ in biological and immunological potencies and may indicate a cellular basis for the molecular heterogeneity of secreted forms of hormones.

This review provides just a few examples of how plaque assays can be used to quantify hormone release from single cells. These assays can also be used in more qualitative applications, such as the identification of a hormone secretor for further study in the living state^{12,13}. By combining variations of plaque assays with other contemporary methodologies, we should be able to address new levels of questions in cell biology.

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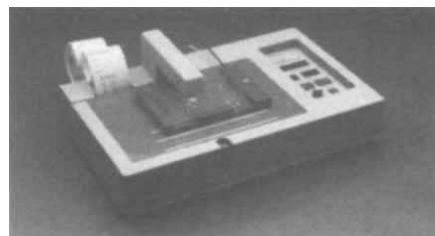
LAST week at the ASBC convention, an **automated microplate reader** from Perkin-Elmer made its market debut (Reader Service No. 100). The Lambda Reader can perform a variety of colorimetric measurements on microscale (less than 300 μ l) samples. Its capabilities span enzyme-linked immunoassays (ELISA), protein determinations, antibody sensitivity tests and complement fixation analyses. Perkin-Elmer's instrument has a wave-length range of 380 to 750 nm and, the company claims, 1 per cent linearity for absorbance 0 to 2. The microprocessor-controlled fixed wavelength spectrophotometer will give the absorbance of samples in 96-well microplates or micro-well strips within 60 seconds. With an alphanumeric touch panel, the Lambda Reader allows flexibility in data entry, wavelength selection, single or multiple



Measurements for microscale samples.

blanking maps, reading sequences, kinetics and data reduction. There are also several options, such as cartridge programability, which expand the \$7,895 (US) reader's potential applications. The user, however, must supply an Epson printer to render the data in an "easy-to-recognize" format.

A **manual microplate reader** with a built-in printer and RS-232C computer interface comes from Cambridge Technology (Reader Service No. 101). The model 700 densitometer specializes in EIA/ELISA operations to an absorbance of 2 with 1 per cent accuracy, and up to A 3.2 with 2 per cent accuracy. The manufacturer supplies filters for 405 and 490 nm wavelengths, but other filters are available and the machine's capabilities extend from 400 to 700 nm. A spring-loaded detent locks in on each well positioned for measurement to aid in correct alignment. The model 700 continuously displays absorbance and well location, but a separate record button must be pressed to send each reading to the printer or an external computer. Cambridge sells the reader for \$2,600 (US). It accepts rigid 96-well plates



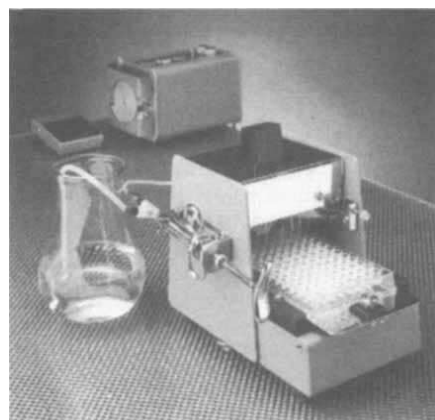
Cambridge Tech's manual reader.

and can adapt to flexible or removable-well varieties with the help of an accessory.

Anyone who buys Labsystems' Uniskan vertical plate reader for enzyme immunoassay (EIA) will also get a **software package** and computer interface cable for free (Reader Service No. 102). The two-part program, which is compatible with the BBC model B, IBM models or IBM 'clones', has provisions for both plate reading and data analysis. The software instructs plates to be read row-by-row or column-by-column; control wells can be positioned randomly, and empty wells can be passed over. Data gets written on a disk from which it can be recalled for threshold, range and standard curve analyses. Labsystem's software also caters to semi-log, log and linear curve preferences in analysis. Written in BASIC in conjunction with a faculty member at Leicester Polytechnic, the program can be customized.

Dispensing devices

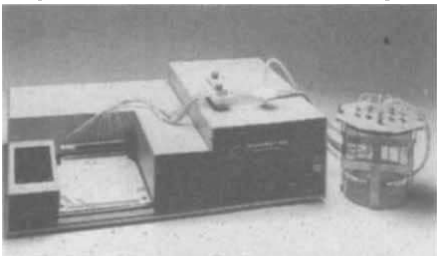
A dispenser from Sterilin, designed for **repetitive multi-well plate procedures**, can carry out filling, inoculation and aspiration operations on all types of standard 96- and 24-well plates (Reader Service No. 103). Sterilin says the Microplater is suitable for numerous sterile and non-sterile applications including cell-washing. Once



Sterilin has relief from repetition.

the dispenser head is positioned above the wells, volumes from 25 to 250 μ l per well can be delivered using the 2 ml syringe assembly that comes with the Microplater. A peristaltic pump manufactured by Sterilin will extend the volume range. The company markets Microplater for £530 in the United Kingdom.

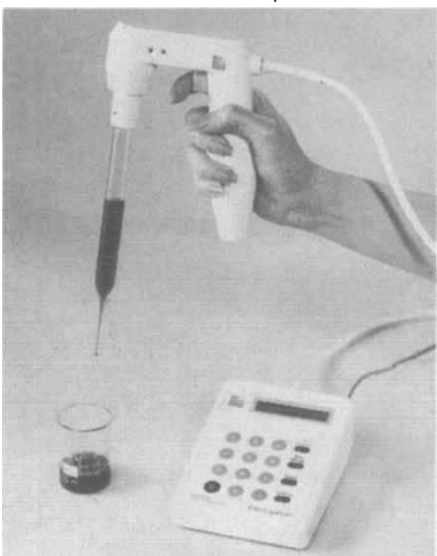
An outgrowth of their manual **reagent dispensing system**, Dynatech's fully automated AM83a Auto Dynadrop system can dispense up to 12 different reagents simultaneously in microplate or strip-well formats (Reader Service No. 104). The latest Dynadrop will fill 8 or 12 wells at a time, depending on user needs, in volumes ranging from 10 to 200 μ l. Dynatech says their system guards against reagent contamination with a design that maintains integrity from the reservoir through to the dispense manifold. The plate carriage on



Room for a dozen reagents.

Dynatech's £3,115 (UK) instrument moves via step-less motor drive, but the automatic mode can be halted with a manual over-ride to allow partial plate preparation.

It looks a bit like a pipetting aid, but the Electrapette from Horwell Ltd uses keyboard control and inexpensive ungraduated pipettes to **automatically dispense volumes** between 100 μ l and 12 ml



Electrapette set with speed control.

(Reader Service No. 105). Set on automatic or manual mode, the Electrapette can register different fill volumes and dispense volumes independently; dispense



Labsystems provides complimentary software for their vertical plate reader.

volumes can only be set to the nearest 10 μ l. Horwell's automatic pipettor also sounds a warning and stops if residual volume in the pipette is insufficient to meet the programmed dispensing volume. The £495 (UK) Electrapette also has a calibration pipette to tailor the Electrapette system to individual liquids.

Micromedic Systems, Inc. increased the potential of their **automatic pipette** by providing it with RS-232C computer interface capability (Reader Service No. 106). The Digiflex-CX can be operated from its own



PC power hooks up with Digiflex

keyboard, or the keystrokes can be entered into a personal computer program that will store and run protocols more complex than the keyboard control accommodates. Micromedic's instrument is precise to 0.5 per cent or 0.1 μ l standard deviation, whichever is greater. The Digiflex gives readouts in microlitre volumes, and can automatically sense the size of installed syringes, a feature that Micromedic calls unique. The base unit costs \$4,300 (US) without the PC.

The tissue section

Reichert-Jung's ergonomically designed **sectioning apparatus** has several interesting features and is available in different versions geared to different budgets (Reader Service No. 107). The company say their Cryostat Frigocut 2800 has a low

build that makes operating the machine more comfortable. The Frigocut also incorporates a non-rusting microtome, the Autocut 2040. Housed in a freezing chamber with a heated, mist-free window, the



Frigocut keeps cool to -40°C .

Autocut can be adjusted to a microfeed mode (for thicknesses between 0.5 and 60 μ m) or a coarse feed for greater thicknesses. Reichert-Jung's instrument starts at £8,000 (UK). It can automatically defrost, or adhere to a programmed 24-hour timer.

For specimen freezing, an **immersion cooler** from Neslab Instruments can serve as an alternative to dry ice and liquid nitrogen at temperatures reaching down to -75°C (Reader Service No. 108). The CC-70W cooler is compact — Neslab



A compact cooler from Neslab.

made it 60 per cent smaller than any other cooler in their line — for especially crowded quarters. The company is offering their new addition at an introductory price of \$495 (US). Its intended uses stretch from vapour trapping and lyophilization to crystallization studies.

Histoline Inc. and Wilkinson Sword Ltd crossed expertise to produce a **disposable microtome blade** that, they say, enhances the consistency of tissue sectioning and improves edge strength (Reader Service No. 109). The new blade, designed for cutting paraffin-embedded human and animal tissue, has a stropped edge free



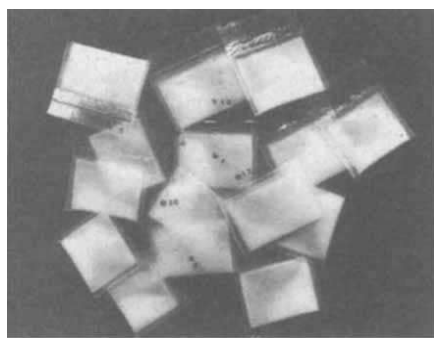
Microtome blades a cut above.

from grind marks. A triple coating of chromium, chromium nitride and PTFE (polytetrafluoroethylene) imparts strength, wear resistance and corrosion resistance and minimizes friction, according to the companies. A pack of 50 blades cost £40 (UK).

Immunochemicals

Three separate series of **immunohistology kits** have emerged from the laboratories of Bio-Yeda Ltd over the past few months (Reader Service No. 110). The first set was created for the location and identification of human tissue components in routinely-fixed paraffin-embedded sections. These kits targeted human immunoglobulins IgA, IgC, IgM, kappa and lambda. Bio-Yeda is currently producing a second group of kits for desmin, prostatic acid phosphatase, insulin, myoglobin and beta human chorionic gonadotropin. The third group of kits, for intermediate filaments, is still in the works. All the company's kits use the avidin-biotin immunoperoxidase staining technique and provide all the reagents needed for that protocol.

A radioimmunoassay kit for **sexual hormone binding globulin (SHBG)** could be valuable in the diagnosis of sex hormone-related pathological conditions (Reader Service No. 111). Metachem Diagnostics Ltd. say they have a highly specific assay with sensitivity of 5 µg per litre that takes less than 3 hours to complete. SHBG is known to influence the biological activity of circulating androgens. The RIA kit, priced at £135 (UK), includes anti-SHBG, ¹²⁵I-SHBG, double antibody-PEG, 7 standards of concentrations from 1 to 400 µg per litre, and high and low controls.

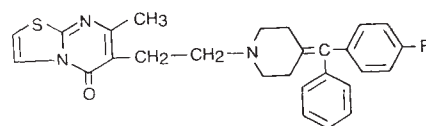


Biosearch's express delivery proteins.

A California-based company is offering **low-cost peptides** that it says it can provide within weeks of ordering (Reader Service No. 112). A technology developed at the Research Institute of Scripps Clinic has given Biosearch, Inc the capability to make hundreds of polypeptides simultaneously, the company says. Biosearch predicts that one of the more useful outgrowths of this technology will be the increased availability of peptide analogues for testing of optimum biological or immunological activity.

A serum **immunoassay for soluble T cell markers** gives a new measure of overall immune system activation (Reader Service No. 113). The Cellfree interleukin-2 receptor test kit from T Cell Sciences determines quantitatively the interleukin-2 receptors (IL-2R) shed from T cells in human sera or culture supernatant. The company's kit allows non-invasive evaluation of activated T cells in organs and tissue. A Cellfree kit uses a sandwich immunoassay with two non-competing monoclonal antibodies, and the results are read colorimetrically from standard 96-well microtiter plates. For \$439 (US), T Cell's kit supplies monoclonal antibodies, a full set of standards, diluent, substrate and microplate.

Calcium, a favourite candidate for the second messenger in signal-transducing systems, may now have to vie with **diacylglycerol** for that position. This lipid, according to Janssen Life Sciences Products,

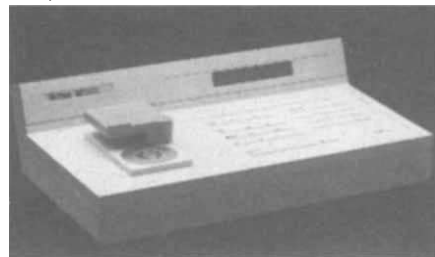


The formula for diacylglycerol kinase inhibition.

has become a contender because elevation of cellular diacylglycerol levels accompanies the phosphoinositide turnover spurred by extracellular stimuli. Janssen now provides an inhibitor to diacylglycerol kinase which potentiates the second messenger effects of diacylglycerol (Reader Service No. 114). Janssen sells 50 mg of R59022, as they call this inhibitor, for £45 (UK).

For the count

Du Pont's biotechnology division last month introduced a **compact automated radioisotope counter** that takes up only a square foot of bench space (Reader Service No. 115). The Bench-Count detector quantitates ³²P and ¹²⁵I radiolabelled samples without using liquid scintillation cocktails. Du Pont expect their instrument to streamline nick translations, end-labelling, iodination, phosphoprotein analysis and other procedures by giving count data during experiments, rather than imposing long waits for results. Because of its small



Results on the spot from Du Pont.

size, the \$2,495 (US) microprocessor-based Bench-Count ought to be able to sit within close range of the experimental procedure itself.

The Mighty Small Deca-Probe is Hoefer Scientific's answer to membrane-cutting for **separate immunotransfers** (Reader Service No. 116). Their new instrument can stain 10 immunotransfers on a single, intact 8 × 8 cm membrane. The membrane fits between two acrylic plates



Multiple transfers, minimal membranes.

which are clamped together and sealed. Parallel troughs, milled in one of the plates, serve as incubation chambers isolated from their neighbours by silicone rubber gaskets. Each trough requires about 0.5 ml of solution, and when staining is complete, the sample lanes can be compared side-by-side. Hoefer ask \$195 (US) for their system, complete with reagents and apparatus. □

These notes are compiled by Karen Wright from information provided by the manufacturers. To obtain further details about these products, use the reader service card bound inside the journal.